EXPERIMENTAL CELL RESEARCH

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THE NATURE OF ISOLATED BACTERIAL FRACTIONS AS RELATED TO PREPARATIVE TECHNIQUE

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Received January 3, 1958

In recent years much effort has been expended towards the goal of preparation of enzymatically active "particulate" fractions from a variety of bacteria. In this area particulate preparations include an electron-transporting particle prepared from *Azotobacter vinelandii* by Bruemmer *et al.* [3], a particulate fraction capable of phosphorylation (Rose and Ochoa [11]). From *Escherichia coli* a cytochrome containing particulate fraction capable of a limited number of oxidative activities has been described (Asnis *et al.* [2]). *Saccharomyces cerevisiae* has been utilized as source material for a particulate fraction capable of lipogenesis (Klein and Booher [6]). Milman and Darter [8] have reported the localization of enzymatic activity as a function of particle size in the organism *Mycobacterium tuberculosis.*

In general, the approach utilized for the preparation of "active" fractions has been disruption of the parent cell by sonic oscillation or mechanical grinding, followed by differential centrifugation. Another useful technique for cellular fractionation stems from the work of Weibull [12] on protoplast formation and subsequent protoplast lysis of *Bacillus megatherium.* The technique has been recently extended to the gram negative organisms by Repaske [9].

A third technique of possible value is that dependent on the osmotic rupture of bacterial cells, proposed by Robrish and Marr [10].

In view of the obvious usefulness of techniques of bacterial lysis and subsequent fractionation, it is desirable to examine critically the nature of preparations obtained by the application of techniques currently in use. This paper reports on the nature of such fractions as determined by electron microscopy.

METHODS

The organism used for these studies was *Azotobacter vinelandii* strain 0. Cells were grown in Burk and Lineweaver's [4] nitrogen-free mineral salt medium with 3 per cent sucrose, 1 ppm Mo added.

The sonicated preparations examined were obtained by suspension of cells in 0.85 per cent NaCl 0.01 *M* tris pH 7.4 and sonication in a 10 Kc Ratheon Sonic Oscillator at maximum intensity for a period of 10 minutes.

The cell membrane fraction of *A. vinelandii* was secured using the method suggested by Repaske [9].

Cell envelopes were obtained by the method of Robrish and Marr [10].

In each case two cellular fractions were examined; the pellets secured on centrifugation at 25,000 $\times g$ for 30 minutes (25p30), and those obtained by subsequent centrifugation at 144,000 for 1 hr. (144p60).

Samples of the preparations were placed on Formvar covered 200 mesh grids, washed with distilled water and allowed to dry. Specimens were fixed for electron microscopy by exposure to osmium tetroxide vapors for 5 minutes. The grids were shadowed at an angle of 1:4 with metallic platinum, and examined in a modified RCA EMU 2b electron microscope.

RESULTS

Each of the low speed fractions was found to be heterogeneous in nature, particularly the 25p30 fraction obtained from sonicated bacteria. This fraction (Fig. 1) consisted of cell wall fragments and non-descript proteinaceous material. Particulates were occasionally noted. Large electron dense structures 0.2–0.4 μ in diameter reported in the 25p30 fraction of Alexander and Wilson [1] were observed in some preparations of this fraction. Bacterial nuclei or cytoplasmic membranes were not identifiable.

The membrane fraction (Fig. 2) obtained by osmotic lysis of protoplasts of *A. vinelandii* consisted of two components. Easily recognizable are flattened disks scattered throughout the field, and identified as membranes. These exist in varying stages of completeness and degradation. Scattered throughout the field and occasionally in close association with the membranes are numerous large spherical granules. Fig. 2 shows a partially degraded membrane with several adherent spherical particles. The variation in particle size observable is considerable.

The cell envelope fraction (Fig. 3) is again contaminated by numerous spherical particles. Fig. 3 shows a flattened bacterial cell most of the internal contents of which have been lost. Despite this, most bacterial cell envelopes appear relatively complete and it is usually possible to distinguish the outer edge of the cytoplasmic content within the flattened bacterial cell wall.

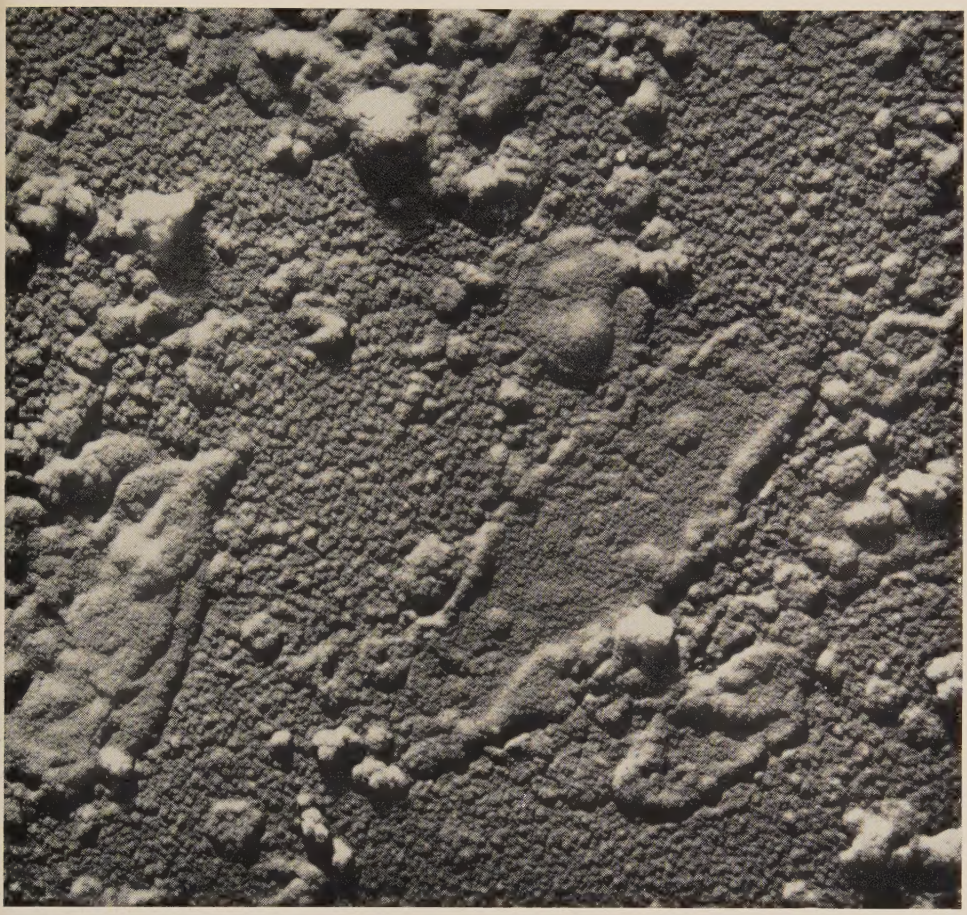


Fig. 1.—25,000 × *g* pellet obtained after 30-minute centrifugation of sonically disrupted *Azotobacter*. The fraction is heterogeneous, a cell wall fragment is visible in the center of the field. × 49,000.

The 144p60 fraction obtained by further centrifugation of the 25p30 supernatant from sonically disrupted microorganisms is illustrated in Fig. 4. This fraction is made up of regularly appearing particulates having a diameter of about 20 mμ. Some aggregates are evident.

The high speed bacterial membrane supernatant 144p60 fraction (Fig. 5) is again particulate. In the preparation illustrated aggregation has proceeded to a considerable degree, and individual particles are less readily resolvable.

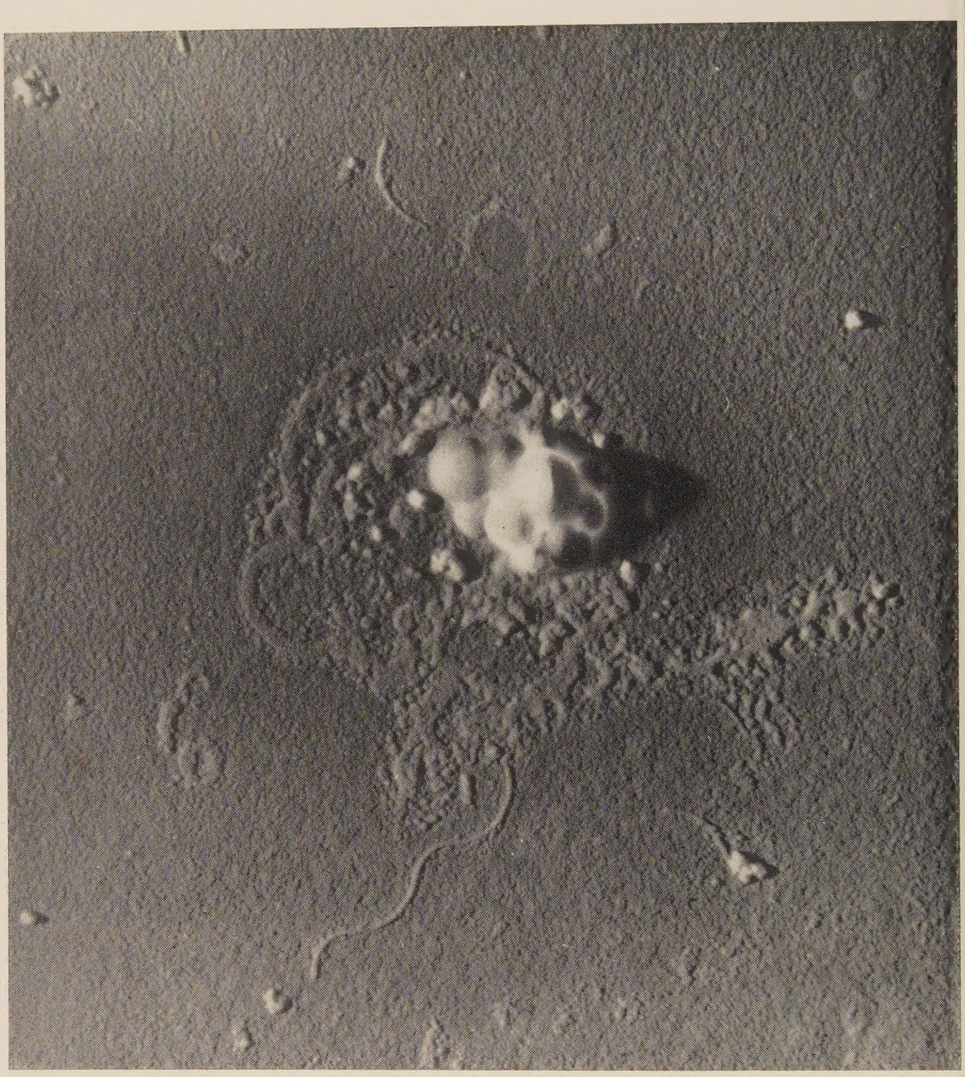


Fig. 2.—Cell membrane fraction of *Azotobacter*. Visible are fragments of bacterial flagella, a partially denatured membrane and adherent spherical bodies. These latter are a common contaminant of the fraction X. ×22,000.

The cell envelope supernatant 144p60 fraction (Fig. 6) appears quite different. In this case, whilst a few ill defined particulates are visible, the main body of the specimen consists of long fibrous strands of material.

Fig. 3.—Cell envelopes of *Azotobacter* secured by osmotic lysis of whole cells. The extracellular spherical particle is representative of the fraction contaminant. ×45,000.

DISCUSSION

In assessing these electron micrographs, it is well to remember that specimen preperation for electron microscopy may result in considerable change in physical appearance. The initial stage of specimen preparation, that of centrifugation, may also induce artifact. In the latter instance partial proteolysis of the specimen may occur during the centrifugation process, resuspension of the resultant pellet may be only partially complete leading to a

final image of component aggregates on electron microscopic examination. Subsequent handling, particularly specimen drying with attendant severe surface tension change, may also result in artifact.

The nature of the three 25p30 fractions examined has been described. Of the three, the sonicated preparation appears the most heterogeneous, and while this fraction may be quite reproducible from an enzymatic point of view, it may be of limited value in determining the nature and biological function of specific entities of the bacterial cell.

The bacterial membrane fraction is extensively contaminated with large spherical particles, and care should be taken to remove this contaminant before ascribing biochemical activity to the membranes. A further disadvantage of this fraction for biochemical study is the fact that the method of preparation involves the use of Versene. As a highly effective chelating agent this substance may, at least partially, remove from the membranes functional metallic ions, thereby serving to mask or decrease inherent enzymatic activity in *in vitro* experiments involving this fraction.

Most promising from a cytological standpoint is the cell envelope fraction. Advantages here are a comparative ease of preparation, the certainty that all bacterial cells present in a sample are exposed to the same disruptive influences, and the isolation of a cellular fraction with considerable cytological organization. As with other fractions under discussion large spherical particles are a contaminant.

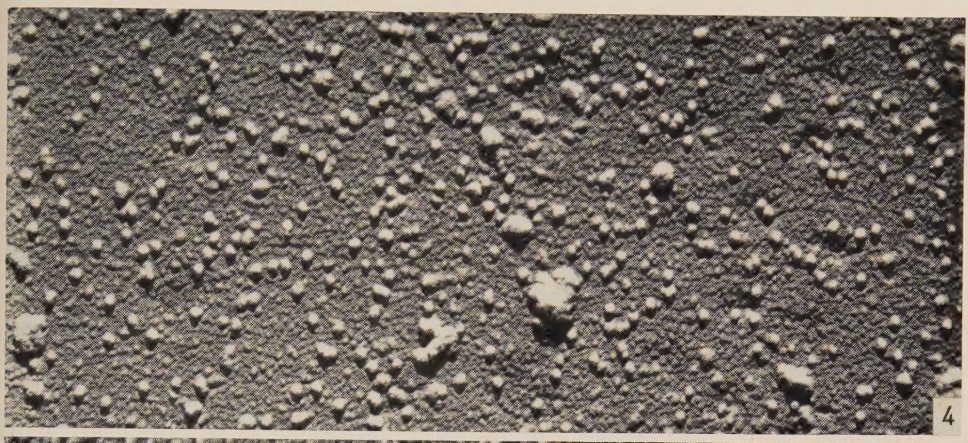
The evidence that the large particulates visible in the electron micrographs presented are discernible components of living cells is minimal. Optical microscope observation of living cells indicates the occasional presence of intracellular granules which are within the appropriate size range. The occurrence of this component, tentatively identified as metaphosphate granules, varies from culture to culture. The frequent occurrence of particulates in the appropriate bacterial fractions is a constant feature, supporting the viewpoint that these particulates are representative of the cellular cytoplasmic areas which appear homogeneous on optical microscopic examination of living cells. One possibility as to the true nature of the contaminant large spherical particles stems from the work of Weibull [13] on *Bacillus megatherium*. Weibull noted the presence of large particulates in low speed centrifugal fractions obtained from disrupted *B. megatherium*. He was able to identify these par-

Fig. 4.—Sonically disrupted bacteria. 144p60 fraction.

Fig. 5.—Bacterial membrane supernatant. 144p60 fraction.

Fig. 6.—Bacterical cell envelope supernatant. 144p60 fraction.

Figs. 4–6 are × 44,000.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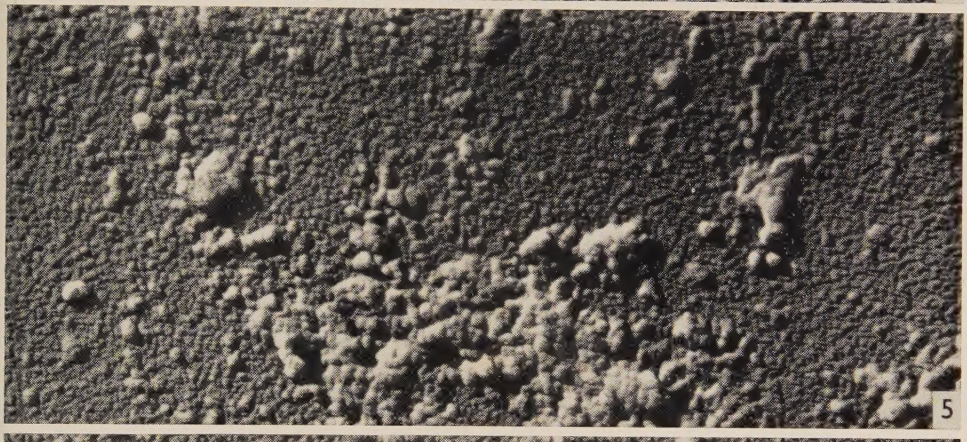
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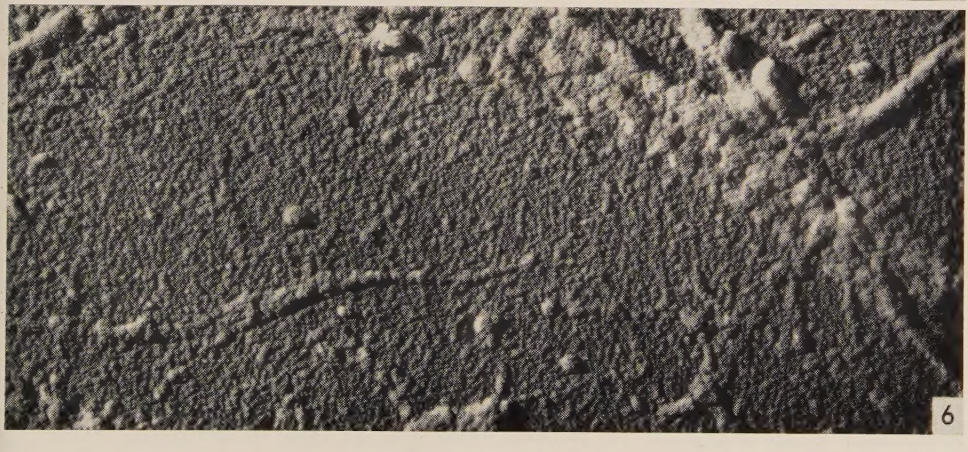
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ticulates as being lipoidal in nature. In view of the size and shape differences, it seems unlikely that the contaminant large particles observed in this sequence of electron micrographs are identical with the electron dense granules of a wide range of sizes, shapes and distributions and of general diameter 30–100 mμ, which have been observed in whole *Azotobacter* cells by Hartman, Brodie, and Gray [5].

The 144p60 fractions examined are not directly comparable. The 144p60 sonicated preparation, as has been succinctly pointed out by Marr and Cota-Robles [7], is made up of cytoplasmic constituents liberated during cell disruption, and a fraction derived from cell wall breakdown. Presumably the 144p60 membrane supernatant fraction represents cytoplasmic material and solubilized cell wall material, while the 144p60 cell envelope supernatant fraction represents cytoplasmic content alone. Differences in appearance of these fractions may be partially ascribed to differences in nature of constituent components and differences in preparative techniques. Of the three, the 144p60 cell envelope supernatant fraction is most likely to result in the preparation of cytoplasmic component materials free of cell wall and cytoplasmic membrane materials.

Ultrathin sections of *Azotobacter vinelandii* fail to reveal the presence of large particulates, other than occasional metaphosphate granules, or of small spherical particles of about 20 mμ diameter noted in this study, by Alexander and Wilson [1], and by Hartman, Brodie and Gray [5]. One must question whether these structures exist in living bacterial cells, or whether they result from the cumulative effects of isolation procedures subsequent to cell breakage.

SUMMARY

The nature of bacterial fractions obtained by three methods of cellular disruption and subsequent differential centrifugation is described. Of the methods examined, cellular fractionation based on the method of osmotic rupture appeared most satisfactory from a cytological viewpoint.

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ELECTRON MICROSCOPE STUDIES OF *SAINTPAULIA IONANTHA* WENDL. POLLEN WALLS

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Received January 3, 1958

The ontogeny of the pollen grain, especially the development of its characteristic wall, poses a number of basic problems in the cytology of growth. Among these are the relationship between pollen wall formation and ploidy or genetic control, and the degree to which the protoplasm is involved in pollen wall development. The pioneer work of Fernández-Morán and Dahl [11] demonstrated that the submicroscopic structure of pollen grain walls can be effectively studied by means of the electron microscope. Therefore, the electron microscope was employed in the present investigation to study the structural development of the pollen grain walls of diploid and tetraploid *Saintpaulia ionantha* Wendl. varieties.

MATERIALS AND METHODS

The diploid horticultural variety 'Ionantha' and its autotetraploid 'Ionantha Amazon' [7] were used throughout this study. Punctured anthers containing meiocytes, microspores, or pollen grains were fixed in 10 per cent formaldehyde (initial pH 8–9, established with lithium carbonate) for 12 hours after evacuation and washed in running water for ½ to 1 hour. One per cent osmium tetroxide adjusted to pH 7.4 with an acetate-veronal buffer was employed as a second fixative. The material remained in the osmium for 3 hours after evacuation and was washed in running water for 15–30 minutes. After dehydration with ethyl alcohol, the specimens were infiltrated with a mixture of 70 per cent *n*-butyl and 30 per cent methyl methacrylate, since this mixture was found best for immediate sectioning. The methacrylate was catalyzed with benzoyl peroxide, and gelatin capsules containing the specimens in this solution were heated to 60°C for 5–12 hours. Glass knives and a Porter-Blum microtome were employed for sectioning (cf. [10] for technique review and approximate schedule followed). Observations were made with the aid of an RCA EMU 2 electron microscope.

RESULTS

After meiosis is complete, the meiocyte consists of four nuclei embedded in a common mass of cytoplasm which is surrounded by an extensive pollen

[1] Fellow in Cancer Research of the American Cancer Society.

mother cell (PMC) wall. The intact PMC wall material is very electron translucent and homogeneous throughout. Delimitation of microspores occurs by a cleavage furrowing process apparently involving vacuoles which contain a substance indistinguishable from the PMC wall under the electron microscope (Fig. 1). At this stage no wall bounding in the cytoplasm could be detected.

When cleavage furrowing is completed, the microspore surface becomes studded with narrow projections (columellae), the long axes of which are perpendicular to the surface (Fig. 2). These exine columellae often appear fountain-shaped, their narrow basal portions in contact with the microspore surface. They are similar to the cytoplasm in electron density but usually show no internal detail. The columellae are longest in the center of the mesocolpium (region between two germinal furrows) and become progressively shorter as the germinal furrow areas (colpi) are approached. The gradation of columellar length persists in the mature pollen grain wall. No columellae or other wall elements are present in germinal furrow areas at this stage. A thin membrane (mesine) appears around the cytoplasm, separating it from the columellae. Rowley [14] used the term "mesine" as "the electron-dense laminated material between the intine and endexine".

At slightly later stages of development, elaboration of the young microspore wall involves the development of the ektexine tectum which connects the distal ends of many columellae (Fig. 8), the formation of a continuous layer (endexine) connecting the columellae bases (Fig. 8), and the increase in thickness of these as well as the ektexine columellae. No exine was observed on the germinal furrow areas during this phase of development (Fig. 8). The exine lumina often contain a moderately electron-dense, granular-striate substance which later disappears.

The development described above takes place within the PMC wall. Thus, the basic architecture of the wall in the mesocolpium is established prior to direct contact with the anther fluid. Although the PMC wall gradually becomes thinner and disappears, the writer's observations indicate that its electron properties do not change. The PMC wall remains in intimate association with the exine as the microspores enlarge. As the investing PMC wall disappears and the microspores lie free within the anthers, wall forma-

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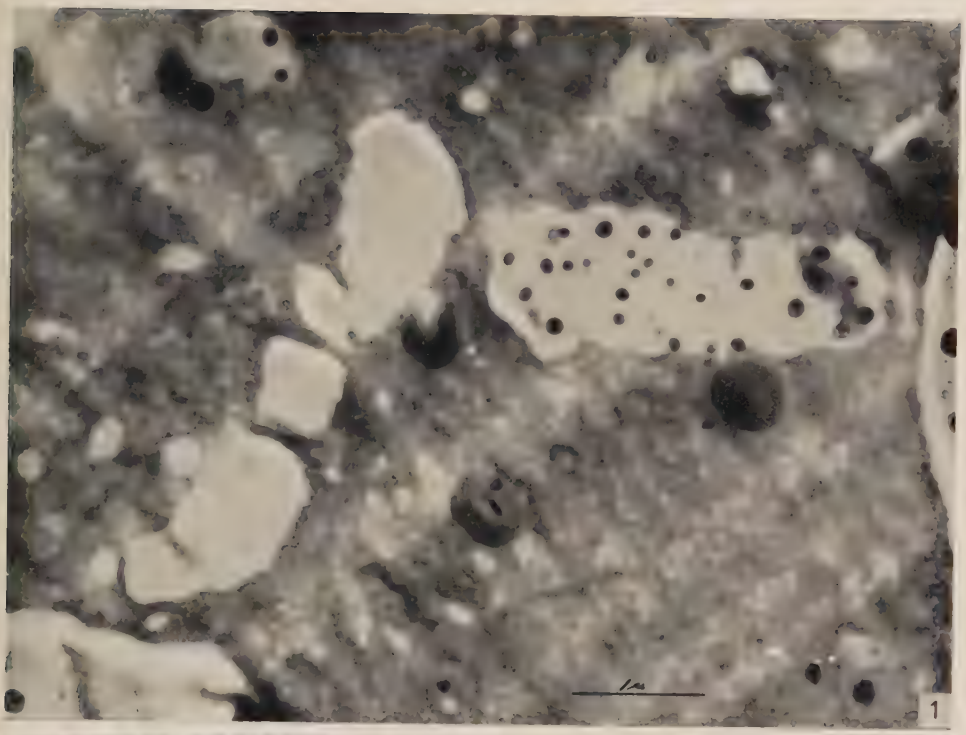
Figs. 1, 3.—Tetraploid variety. Fig. 2.—Diploid variety.

Fig. 1. Cleavage furrowing in PMC showing vacuoles delimiting portions of 3 microspores. PMC wall at lower left and extreme right. ×17,550; *Fig. 2.* Wall initiation in young microspore showing exine columellae, thin mesine at cytoplasm periphery. Left background is PMC wall. Note portion of nucleus and double-layered nuclear membrane (arrow). ×17,550; *Fig. 3.* Portion of older microspore in mesocolpium and margin of germinal furrow (top). *t*, Tectum; *c*, columella; *en*, endexine; *m*, mesine; *in*, intine. PMC wall absent. ×17,550.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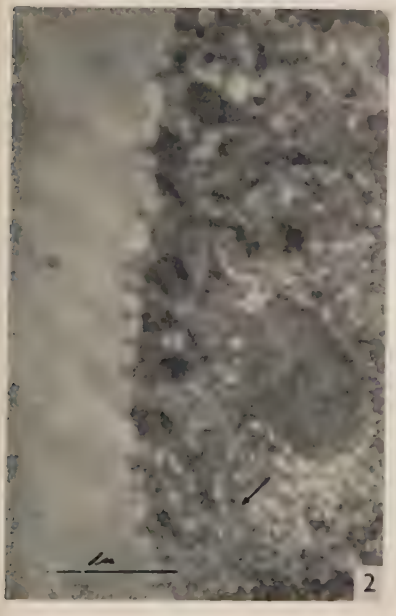
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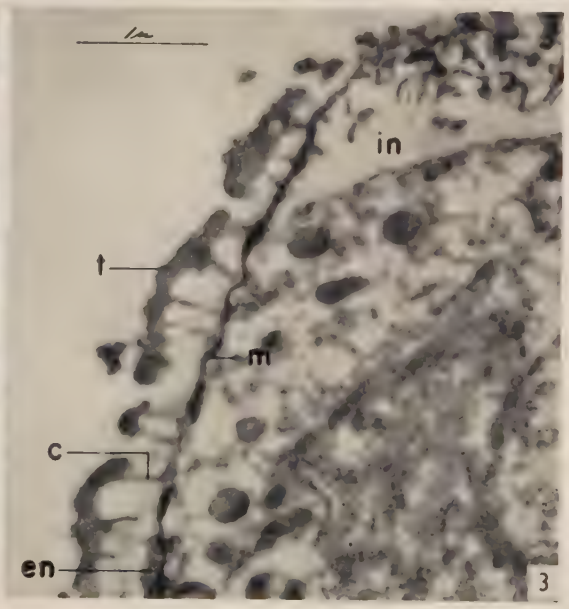
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tion in the germinal furrows has begun. The basic structure of the furrow wall is complete in the older microspores (Fig. 3).

The mesocolpium exine of the young pollen grain (Fig. 4) is uniformly granular. It is tectate and massive but becomes thinner in both directions toward the furrows. Immediately beneath the mesocolpium exine is a thin, slightly more electron-dense, mesine layer which sometimes shows a lamellar structure during this period of development. The mesine is in contact with the cytoplasm (Fig. 4). The mesine broadens in the furrow areas, often displaying a marked lamellar structure, and its outer surface is now flecked with exine (Fig. 9). A thick intine is also present beneath the mesine in this area and only in this area (cf. Figs. 4 and 9). At this stage the intine is partially composed of an extensive network.

As the pollen grain matures, intine is formed under the mesine of the mesocolpium, and still later an additional but different layer develops over the exine (Figs. 5 and 6). The latter layer, the perine [8], is more electron-dense than the exine itself. In some places it actually appears to plug gaps in the tectum and may line at least a portion of the lumina in the exine. The perine is also present on the furrow exine.

By the time the pollen grain is mature, the intine has been completely formed between the mesine and cytoplasm to establish a continuous wall layer (cf. Fig. 5). The intine is much thinner in the mesocolpium regions (cf. Fig. 5 and 7) than in the furrow areas (cf. Figs. 5 and 10). It is finely granular and very electron-translucent but is permeated, especially in the outer region, with more electron-dense structures whose irregular shapes suggest a coarse network that has been thinly sectioned (Figs. 7 and 10). The mesine remains thin in the mesocolpium and thick in the furrow. It is lens-shaped in both transverse and longitudinal sections of the furrow. The granular exine appears to vary somewhat in electron density (Figs. 7 and 10). Exine is generally thinner in the furrow areas than in the mesocolpium regions (Figs. 5, 7 and 10), and its occurrence as scattered flecks in the furrows apparently represents columellae without a tectum or with an incomplete one. Wall structure is modified in the equatorial region of the furrow where the circular germ pore is located. The exine of the germ pore is thicker than that of other

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Figs. 4–7.—Diploid variety.

Fig. 4. Wall in mesocolpium of young pollen grain. Intine absent. × 22,140; *Fig. 5.* Almost median cross section of nearly mature tricolporate pollen grain. Note decreasing thickness of exine and increasing thickness of intine toward furrows. × 5040; *Fig. 6.* Margin of germinal furrow of nearly mature pollen grain. Exine partially embedded in perine (*p*). × 17,550; *Fig. 7.* Wall in mesocolpium of mature pollen grain. *in*, Intine; *p*, perine remains. × 13,500.

1 μ
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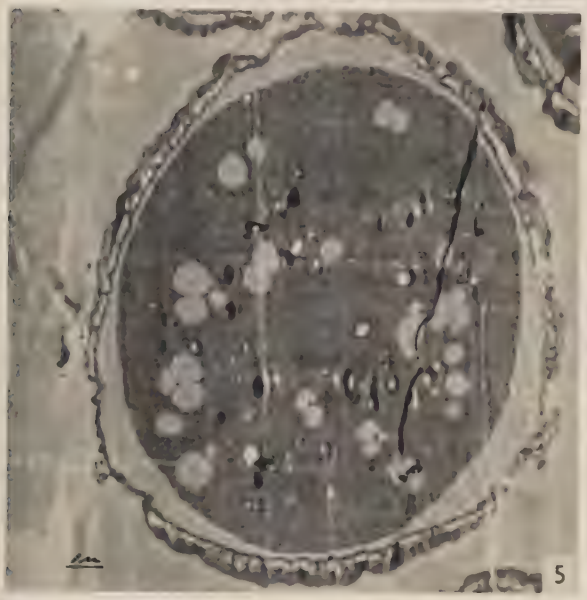
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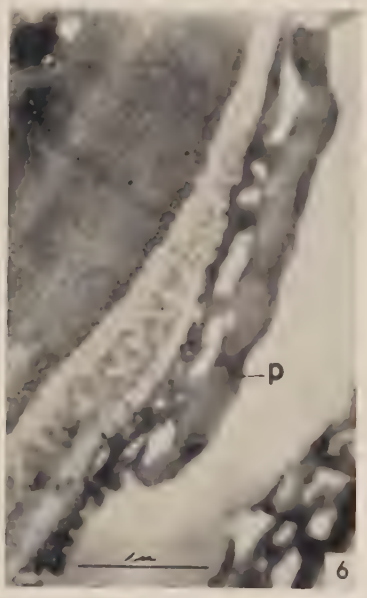
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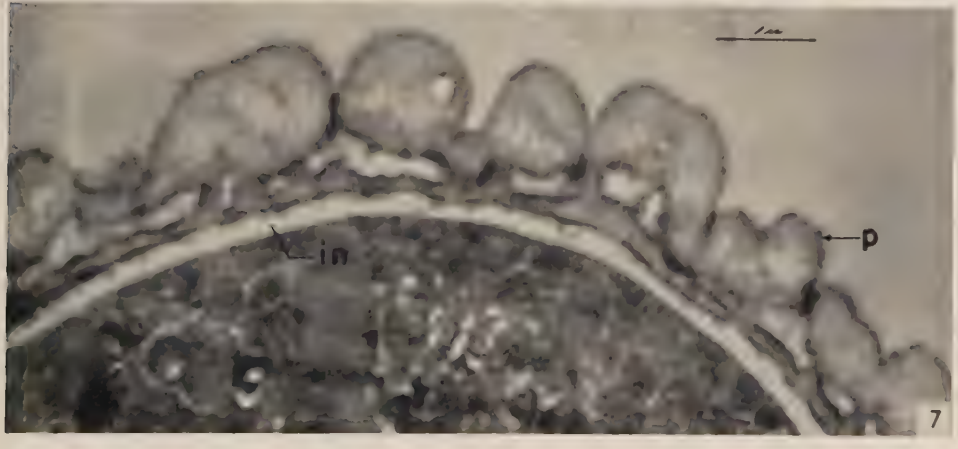
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parts of the furrow. The center of the pore (Fig. 10) consists of a moderately thick mesine layer covered with exine, but the mesine becomes quite thin and exine is usually absent or much reduced at the periphery. The mesine thickens, and exine is once again present in the zone immediately beyond the pore. The perine persists in varying thickness as a blanketing layer over the entire pollen grain.

The basic architecture of the pollen wall is complete before aborted pollen can be recognized. An aborted grain has no intine or protoplast, but it does have mesine and exine layers (Fig. 11). The exine and mesine layers are similar to those of normal pollen, although the exine lumina often do not appear as large as those of normal pollen. Perine covers the aborted grain in the same manner as it covers normal pollen.

Observations of the microspore nuclear membrane indicate the presence of a double layer (e.g. Fig. 2). Numerous mitochondria showing some lamellate structure, an endoplasmic reticulum, and other cytoplasmic structures were noted. Examples of these can be seen throughout the illustrations presented (cf. esp. Fig. 12), but a detailed description of these and their significance must await more exhaustive studies.

The results presented above apply to both varieties of plants studied. Thus, the pollen grain wall development and its structural detail are the same in the diploid and its autotetraploid. Only two exceptions to this statement were found. Pollen grains of the tetraploid are larger, and their walls are thicker; and while pollen from the diploid is tricolporate (three germinal furrows, each with an equatorial germ pore, Fig. 5), both tricolporate and tetracolporate pollen is produced by the tetraploid. The extra germinal furrows in the tetraploid are identical with the other furrows.

DISCUSSION

The ultrastructure of mature *Saintpaulia ionantha* pollen walls agrees, in general, with that reported in investigations of other plants. The blanketing layer (perine) which covers the exine has been observed by Afzelius [2],

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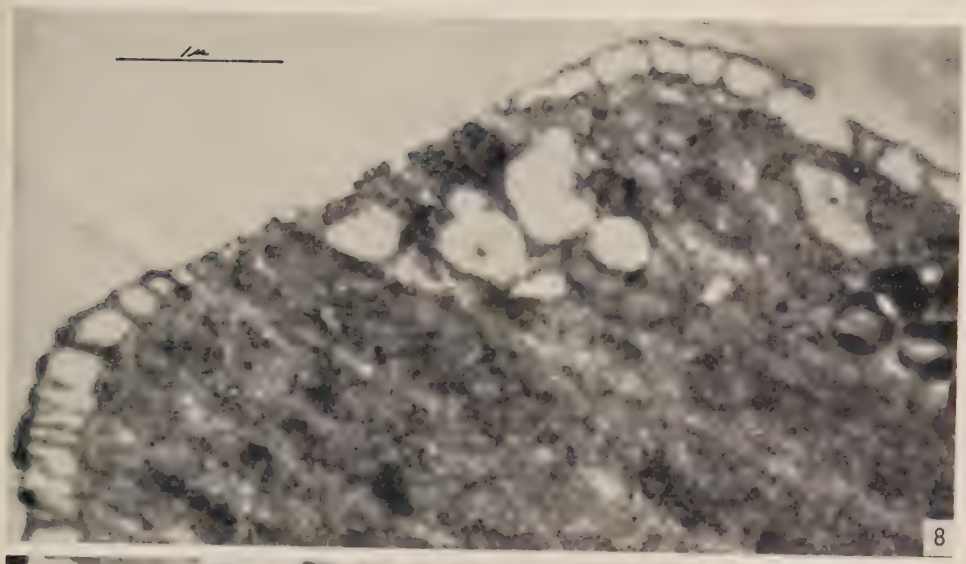
Fig. 8.—Tetraploid variety. Figs. 9–10.—Diploid variety.

Fig. 8. Furrow area of young microspore. Tectum, columellae, and thin mesine present. Endexine beginning to form. No wall formation in furrow. Background at top is PMC wall. × 22,140; *Fig. 9.* Furrow area of young pollen grain (adjacent to germ pore). Tectate exine at margins and intectate in furrow. Thin, lamellate mesine at margins and thick, multi-lamellate in furrow. Under mesine, thick intine (*in*) is permeated by a delicate network extending to cytoplasm below. × 17,550; *Fig. 10.* Furrow area in region of germ pore in mature pollen grain. *m*, Mesine; *p*, perine remains; *in*, intine. × 17,550.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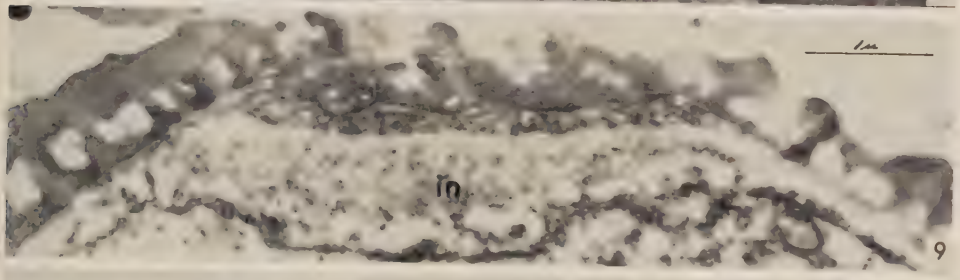
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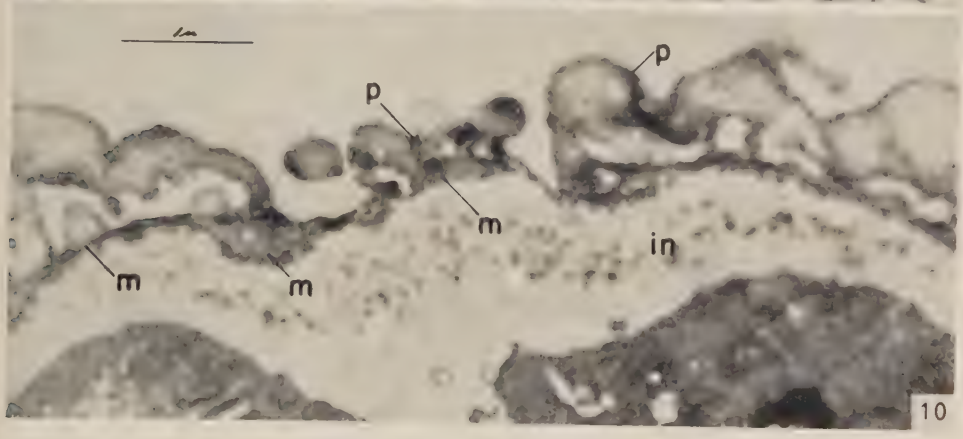
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who described it as granular, and by Mühlethaler [13] and Rowley [14]. Although examples of a granular-lamellar structure in the outer exine have been reported [4, 9] (see also the review by Afzelius [3]), the exine is typically granular-amorphous in section [1, 2, 3, 9, 13, 14]. With the aid of shadowing techniques, Sitte [15] similarly found that exine sporopollenine occurred in the form of spherical particles. However, an electron micrograph made by Afzelius [cf. 9] illustrates "osmium stained channels" in the otherwise granular-amorphous tectum of *Corylus*. The mesine or an analogous layer, when present, is lamellate-granular [2, 3, 14]. The finely granular intine, sometimes appearing delicately reticulate, is permeated by a coarser, net-like structure. Fernández-Morán and Dahl [11] tentatively viewed the intine as a relatively smooth matrix permeated by a system of channels, but Mühlethaler [12] thought it homogeneous in his material. Rowley [14] reported the intine as granular or finely lamellate, but now considers it a network rather than a lamellate system (personal communication, 1957). The occurrence of cellulose microfibrils in the intine was demonstrated by isolation and shadowing techniques [15]. Enlargement of the intine into a lens-shaped thickening under each germinal furrow also has been observed with the electron microscope [12, 14], but a similar thickening of the mesine in this region has not been previously reported.

In a light microscope study, Castetter [6] showed that delimitation of microspores in *Melilotus alba* involved the formation and alignment of vacuoles. Cleavage furrowing proceeded centripetally from the cytoplasm periphery, the advancing furrows cutting through the partitions separating the aligned and progressively enlarging vacuoles. Ehrlich [7] thought that vacuoles may be similarly involved in the furrowing process which delimited microspores in *S. ionantha*, and the present electron microscope study confirms these observations. The vacuolar and PMC wall substances are homogeneous and display similar electron densities. Vacuolar enlargement apparently causes the partitions between vacuoles to rupture, thus dividing the protoplasmic mass into microspores.

It is clear that the basic architecture of the pollen grain wall in *S. ionantha* is established at an early stage. As a result of electron microscope studies, Mühlethaler [12] suggested that exine formation began during meiosis, and

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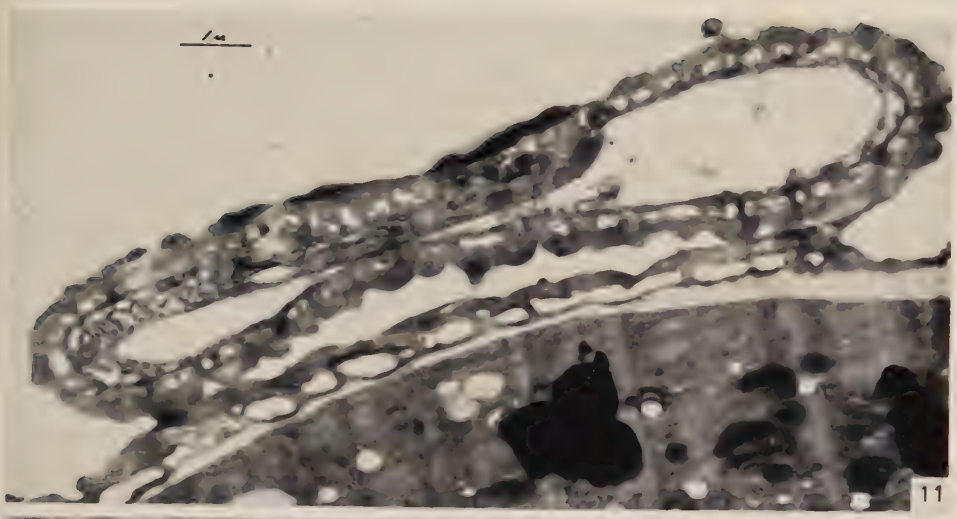
Fig. 11.—Portion of aborted grain (partially tangential section) adjacent to portion of nearly mature normal one. Tetraploid variety. × 9720.

Fig. 12.—Portion of cytoplasm of PMC at interphase II before cleavage furrowing. Clear area at lower right is PMC wall. *mi*, Mitochondrion; *er*, endoplasmic reticulum. Diploid variety. × 44,850.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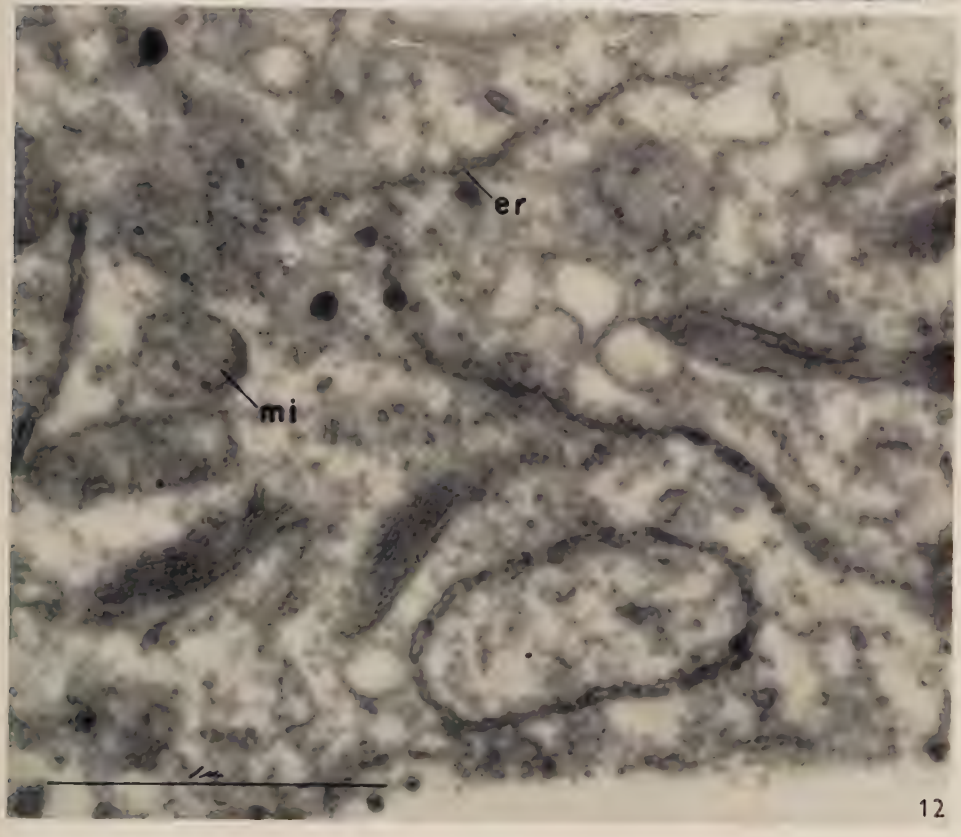
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Rowley [14] demonstrated exine development at late telophase II in *Tradescantia*. Exine and mesine formation in the mesocolpium regions of *S. ionantha* microspores is initiated soon after completion of the cleavage furrowing, and in the germinal furrows somewhat later. Sections of very young microspores often show a finely convoluted cytoplasmic surface. When present, these convolutions are usually associated with the bases of the young exine columellae. Only rarely does a delicate extension of the cytoplasm seem to be involved in the structure of these exine columellae. With the exception of mere physical contact, definitive reports of cytoplasmic association with the exine and mesine are lacking in electron microscope literature. Observations of the intine provide better evidence for a more intimate association between the cytoplasm and the wall. Fernández-Morán and Dahl [11] demonstrated that *Tradescantia* intine was "permeated by irregular, rather sinuous strands that appear to be intimately associated with peripheral cytoplasm". The intine of *S. ionantha* shows analogous structure (e.g. Fig. 9). Investigations providing additional details regarding the cytoplasm–wall relationship are needed for clarification of this important association.

Although the diploid *S. ionantha* studied produces only tricolporate pollen grains, tetracolporate grains are typical of the related tetraploid. The tetraploid also produces some tricolporate and pentacolporate grains, but the latter are rare [7]. Electron microscopy reveals the same development and structure for all germinal furrows in pollen of both the diploid and tetraploid. Thus, duplication of genes through polyploidy does not affect the structural pattern of wall development but does result in variation of the number of germinal furrows. The interesting problem remaining is the manner by which the genetic mechanism of the microspore protoplast operates to provide for a differentiated furrow site with its consequent elaborate and distinctive structure.

After considering the problem of pollen wall development, Beer [5] maintained that "the origin and first differentiation takes place under the direct control of the protoplasm, but, once formed, the further growth may continue and, moreover, maintain throughout the characteristic shape and structure of the part, quite independent of any immediate guidance from the living protoplast, providing only the material necessary for this growth is forthcoming". Experiments specifically designed to determine the source(s) of pollen wall building material and the mechanism by which this material is elaborated into the wall are necessary in order to resolve the existing controversy concerning these points. Some statements which bear upon the problem are in order, however. The basic structure of *S. ionantha* pollen grain walls

is established before abortive grains can be distinguished from normal pollen. Aborted grains, when subsequently found, lack intines and protoplasts but have exine and mesine layers similar to normal pollen. Thus, the intine is not formed without the presence of the protoplast. However, the influence of the protoplast on exine and mesine formation is uncertain, since the stage at which the protoplast ceased to function could not be determined. Rowley [14] reported similar results in an electron microscope study of *Tradescantia*, but his work more clearly indicated that exine elaboration was doubtful after the protoplast ceased to function. In view of the preceding results regarding orderly development of the diversely differentiated features of the pollen grain wall, participation of the protoplast in wall formation seems necessary. The extent to which it is involved and its manner of operation remain to be determined.

SUMMARY

Double fixation followed by standard electron microscopical techniques yielded good results in the preparation of *S. ionantha* meiocytes, microspores and pollen grains for the present investigation. Delimitation of microspores occurs by cleavage furrowing which apparently involves the enlargement of vacuoles between the developing microspores. Columellae are the first exine structures formed in the mesocolpium regions of the microspores, and mesine is found about the same time. The tectum and endexine are formed next but the germinal furrows remain without wall elaboration until later. These stages occur within the PMC wall. Thus, the basic architecture of the *S. ionantha* pollen grain wall is established early in microspore development. The intine is formed later. The structure and development of the germinal furrow differs somewhat from that of the mesocolpium. At maturity, the furrow consists of a thick, laminated mesine which is flecked with exine and underlain by a thick intine. This structure is modified in the germ pore area. The intine first appears in the germinal furrow regions, becomes thickest there, and is the last wall layer to be completed. Appearance of the perine coincides with the final stages of wall development. The wall structure of aborted pollen is similar to that of other pollen except that no intine is found. Duplication of genes by polyploidy does not alter the development and structure of *S. ionantha* pollen grain walls. Variation in the number of germinal furrows and increased wall thickness were the only observed effects of polyploidy.

The writer wishes to express his appreciation to Professor A. Orville Dahl for making his laboratory available for this investigation. He also thanks the Department of Botany and the University of Minnesota for providing additional facilities.

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THE INFLUENCE OF THE ANTERIOR HYPOPHYSIS ON THE MORPHOLOGY AND FUNCTION OF THE ADRENAL CORTEX *IN VITRO*

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One of us (Schaberg [14]) has previously described the results of tissue culture experiments in which the influence of ACTH and explants of the anterior hypophysis on the adrenal cortex of 5-day-old rats was studied. It was shown that ACTH as well as explants of the anterior hypophysis elicit analogous morphological changes in the adrenal cortical explants. Even after a preliminary culture period of some days the anterior hypophysis was still able to elicit these changes. This latter observation seemed to us of some importance as several investigators think that at least a large proportion of the corticotrophic activity of the anterior hypophysis is regulated by the hypothalamus and therefore might be expected to decrease rapidly after contact with the hypothalamus is disrupted [7]. In order to study these observations in more detail we arranged a series of experiments in which we combined cultures of the adrenal cortex with explants of the anterior hypophysis previously cultured for different periods. We have studied not only the morphological changes produced under the influence of the anterior hypophysis but also the amount of corticosteroids released by the adrenal cortex.

METHODS

Tissue culture procedures.—Adrenal glands and hypophyses were obtained from 5-day-old rats. In rats of this age the adrenal is bounded by a well defined capsule and contains both cortical and medullary tissue. The cortex shows two zones: a narrow peripheral zone, the zona glomerulosa, and a wider inner zone, the zona fasciculata. At this stage no zona reticularis is found and the zona glomerulosa consists mainly of small cells which are crowded together without definite organization; a few larger cells are present among the small ones. The zona fasciculata is composed of large, uniform, vacuolated cells arranged in more or less radially directed cords. With the aid of a dissecting microscope the surrounding tissue was removed and the adrenal

explants, which measured approximately 0.06 cmm, were taken from the peripheral part of the cortex. In each a portion of the capsule was present.

The explants of the hypophysis were taken from the anterior lobe only. The hypophysis of the 5-day rat consists mainly of chromophobe cells; there are a few acidophil cells but these have not yet attained full size and staining properties. Small, basophil, non granulated, PAS-positive cells are present mainly along the vessels.

The tissue fragments were placed on top of a coagulated medium and the container was closed with a glass plate dipped in heated paraffin; the cultures were incubated at 37°C [4]. Every third day the explants were removed from the clot, washed in saline and transferred to a freshly prepared medium.

The medium consisted of 1 part of adult human plasma, and 4 parts of brain extract from a human fetus, diluted 10× with Hanks' balanced saline solution. Plasma and brain extract were prepared as described by Gaillard [4], the only difference being that a solution described by Hanks replaced Gey's saline solution [6].

With these methods three groups of experiments were performed (see also Table I).

(1) Explants of the adrenal cortex were cultured for 6 days and then combined with fresh explants of the anterior hypophysis.
(2) Explants of the adrenal cortex were cultured for 6 days and then combined with fragments of the anterior hypophysis previously cultured for 6 days.
(3) Explants of the adrenal cortex were cultured for 6 days and then combined with fragments of the anterior hypohysis previously cultured for 12 days.

After a further culture period of three days the combined cultures were fixed in Bouin's solution and the medium was collected for corticosteroid estimation. The medium of four culture glasses, in each of which three combined cultures had been grown, was pooled. In this way about 0.5 ml of medium was available for the estimation.

In each of these experiments fragments of the adrenal cortex without pituitary fragments and fragments of the adrenal cortex combined with renal tissue served as controls. The medium without any tissue, was also incubated to obtain blank values for our chemical estimations.

The fixed cultures were embedded in paraffin, serially sectioned and stained with hematoxylin and eosin or according to Mallory's azan method.

Corticosteroid estimation.—The spectrophotometric method of corticosteroid estimation as described by Saffran and Schally [10] was adapted to our purpose. Each sample of medium was measured with the aid of a tuberculin syringe with a long needle, and 0.5 ml was shaken by hand for 5 minutes with 3.5 ml chemically pure methylene chloride. The ensuing emulsion was broken up by centrifuging for 10 minutes at approximately 3000 rpm and the aqueous layer removed by suction. If the corticosteroid solution became cloudy again after centrifugation one or two drops of ethyl ether nearly always sufficed to restore clarity. Methylene chloride served as a blank solution; a known standard of hydrocortisone, in one or more concentrations, was always measured along with the unknown samples. The optical densities were read at 235, 240, 250, 255 and 260 mμ; the difference between 240 and 255 mμ served as a measure of the quantity of corticosteroids.

RESULTS

Influence of the Anterior Hypophysis on the Morphology of the Adrenal Cortex

Explants of the adrenal cortex cultured for 6 days and then combined either with fresh explants of the anterior hypophysis or with explants of the anterior hypophysis previously cultured for 6 or 12 days (Figs. 1, 2, 3, 4, 5, 6).—Significant morphological differences were noticed between these adrenal cultures and the controls. There was a considerable variation however in the response of different explants to the presence of the anterior hypophysis. Thus in some cultures the cells showed a slightly decreased nuclear-cytoplasmic ratio and coarse cytoplasm sometimes with small vacuoles (Figs. 2 and 5), while others contained numerous cells with large vacuoles (Fig. 3) enclosing a colloid-like material. Some of the cells with large vacuoles were obviously degenerate; they had ruptured and their nuclei showed clumping of the chromatin. In these experiments the cells immediately adjacent to the pre-existing capsule were also modified by the anterior hypophysis.

We had the impression that the elicited changes were most marked in the adrenal cultures that had been combined with fresh explants of the anterior hypophysis and that the activity of the hypophysis explants which had been cultured separately for some time had decreased during this period. However in several cultures combined with pituitary explants previously grown for 12 days pronounced changes were still seen (Fig. 6).

Fragments of the peripheral part of the adrenal cortex cultured alone and in the presence of fragments of kidney (controls) (Figs. 7, 8, 9, 10).—These explants behaved like those previously described [12]. The tissue fragments cultivated alone (Figs. 7, 8) were encapsulated by outgrowing fibroblasts during the first few days, the zona fasciculata degenerated and regeneration took place from the inner part of the pre-existing capsule and from the cells adjacent to the capsule. After 9 days in most cultures the degenerated areas had been replaced by cells which were indistinguishable from those normally found in the zona glomerulosa. Rarely a few scattered fasciculata-like cells were seen. The adrenal explants cultured in the presence of renal tissue behaved in the same way (Figs. 9, 10).

The Influence of the Anterior Hypophysis on the Release of Corticosteroids

Table I gives the results of the estimations of the corticosteroids in the medium. From these figures it is clear that in these experiments the anterior

hypophysis stimulates the release of corticosteroids, whereas renal tissue appears to have no effect. These differences are statistically significant. The activity of the anterior hypophysis previously cultured for 12 days seems to decrease as compared with that of the fresh explants and of the 6-day-old hypophysis cultures, but this difference is not statistically significant.

TABLE I. *Corticosteroid estimations in the medium.*

Combinations	No. of estim.	Corticost. in γ/cc.
I. Adrenal	13	3.8 ± 0.7
II. Adrenal + Kidney	6	3.2 ± 0.7
III. Adrenal + Hypophysis^{0}	12	8.7 ± 1.7
IV. Adrenal + Hypophysis^{6}	11	8.4 ± 1.0
V. Adrenal + Hypophysis^{12}	9	6.8 ± 1.3

Statistical significance (Wilcoxon): III > I and II ($p_2 < 0.002$); IV > I and II ($p_2 < 0.002$); V > I and II ($p_2 = 0.038$); III > V ($p_2 = 0.92$). Hypophysis^{0-6-12} means: explants of the hypophysis previously cultured for 0, 6 or 12 days.

DISCUSSION

In our *in vitro* experiments the amount of cytoplasm in the cortical cells increases under the influence of the anterior lobe of the hypophysis. In some

Fig. 1.—Explant of the peripheral part of the adrenal cortex cultured for 6 days and then combined with a fresh explant of the anterior hypophysis. Upper half: the hypophysis; lower half: the adrenal cortex with the pre-existing capsule in the centre. × 160.

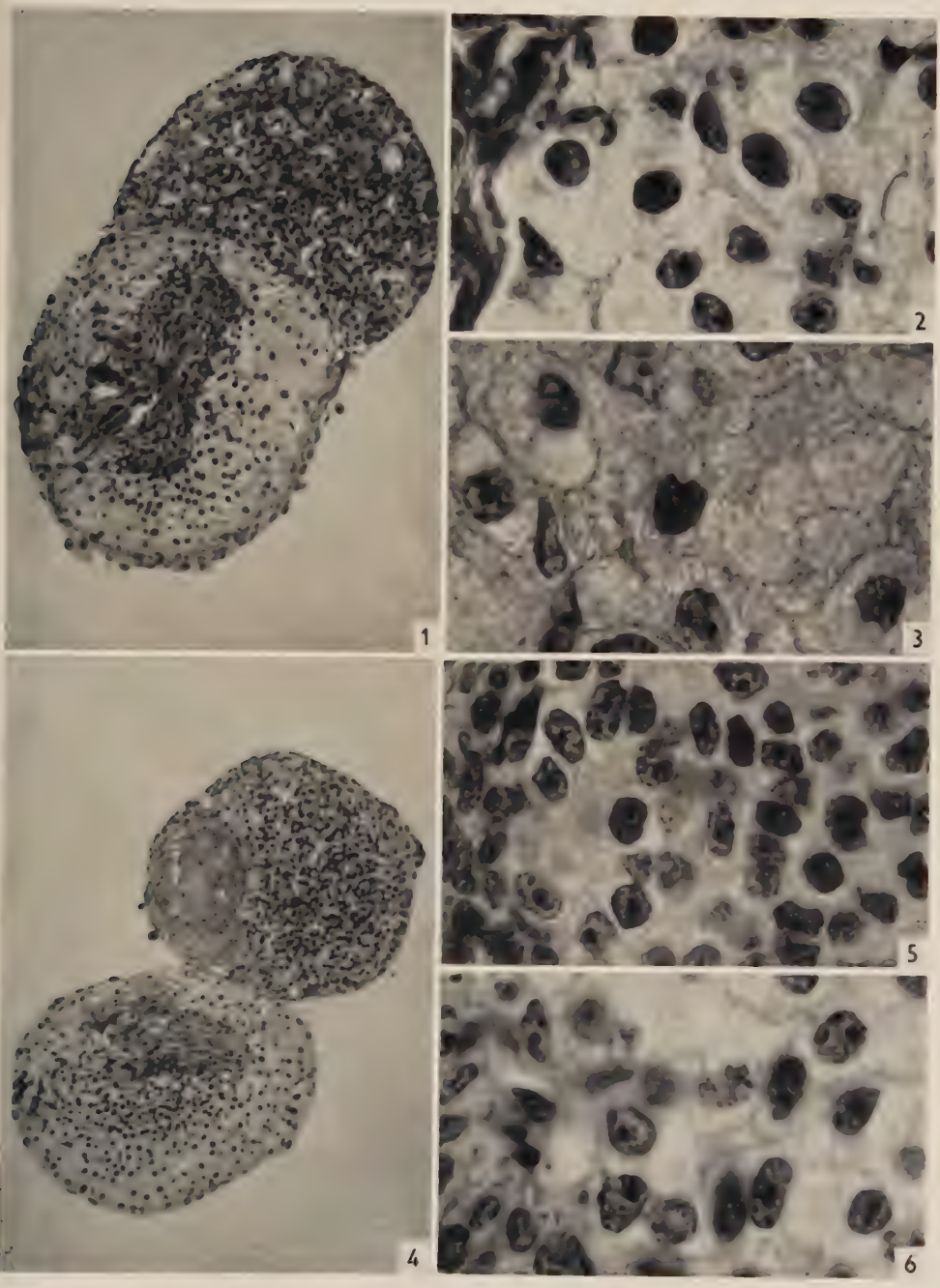
Fig. 2.—Same section as Fig. 1, high magnification of the subcapsular area of the adrenal cortex. There is a marked decrease of the nuclear-cytoplasmic ratio in comparison with the controls. The cytoplasm is somewhat coarse. × 1200.

Fig. 3.—Same section as Fig. 1 and 2, high magnification. Adrenal cortical cells with vacuolisation of the cytoplasm. × 1500.

Fig. 4.—Explant of the peripheral part of the adrenal cortex cultured for 6 days and then combined with an explant of the anterior hypophysis previously cultured for 12 days. Upper half the hypophysis, lower half the adrenal cortex. × 150.

Fig. 5.—Same section as Fig. 4, high magnification of the subcapsular area of the adrenal cortex. The nuclear-cytoplasmic ratio has only slightly decreased in this region. × 1150.

Fig. 6.—Same section as Figs. 4 and 5, high magnification. The nuclear-cytoplasmic ratio has markedly decreased in this part of the subcapsular area. There is some vacuolisation of the cytoplasm. × 1150.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cultures the nuclear/cytoplasmic ratio decreases only slightly and in these the cells have a coarsely spongy cytoplasm. In other cultures there is a definite vacuolization of the cytoplasm and scattered cells show large vacuoles which sometimes rupture and become confluent with those of the adjacent cells; this process leads to cell death. These changes are essentially the same as those observed by Selye and Stone [15] and Wilbur and Rich [17] in rats treated with crude extract of the anterior hypophysis and ACTH. Rich [9], Liebegott [8] and Schaberg [11, 13] observed similar morphological changes in the adrenals of patients who died from an acute disease.

The results of the present study not only confirm the observations previously reported by one of us (Schaberg [14]), but they also demonstrate that explants of the anterior hypophysis stimulate the release of corticosteroids from adjacent fragments of adrenal cortex. It seems unlikely that the increased amount of corticosteroids in the medium is due to an increased release of corticosteroids already present in the cells at the time of explantation. The explants were cultured for 6 days before they were combined with the fragments of anterior hypophysis; during this period the medium was refreshed twice and the cultures were washed in Hanks' solution at the same time. It is more likely that a real corticosteroid production takes place in the cortical cells under the influence of the hypophysis. We think that these experiments also suggest that the observed changes are the morphological manifestation of an extreme stimulation of the cortical cells.

Deane and Greep [3] found that hypophysectomy in rats does not cause significant atrophy of the zona glomerulosa and concluded that this zone functions independently of the hypophysis. Bergner and Deane [2] could not affect this zone with ACTH, but Baker [1] showed that with increasing adrenocorticotrophic stimulation all of the cortical cells in the region of the zona glomerulosa resemble those of the zona fasciculata. In our *in vitro* experiments the cells immediately adjacent to the pre-existing capsule were

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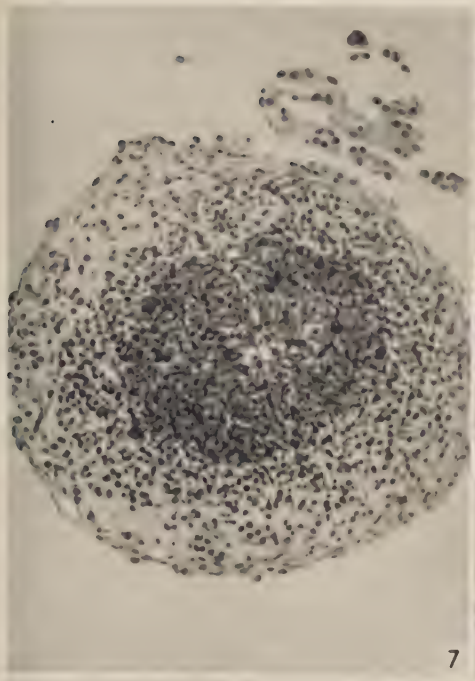
Fig. 7.—Explant of the peripheral part of the adrenal cortex after cultivation for 9 days. The central part shows the pre-existent capsule. × 160.

Fig. 8.—Same section as Fig. 7, high magnification of the subcapsular area. The cells have only a little cytoplasm. × 1200.

Fig. 9.—Explant of the peripheral part of the adrenal cortex cultured for 6 days and then combined with a fresh explant of the kidney. The upper half shows the renal tissue, the lower half the cortical tissue. × 180.

Fig. 10.—Same section as Fig. 9, high magnification of the subcapsular cortical tissue. These cells also have little cytoplasm. × 1200.



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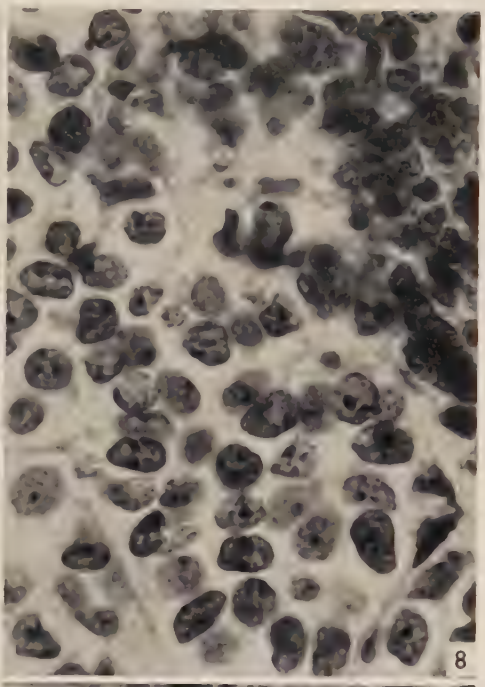

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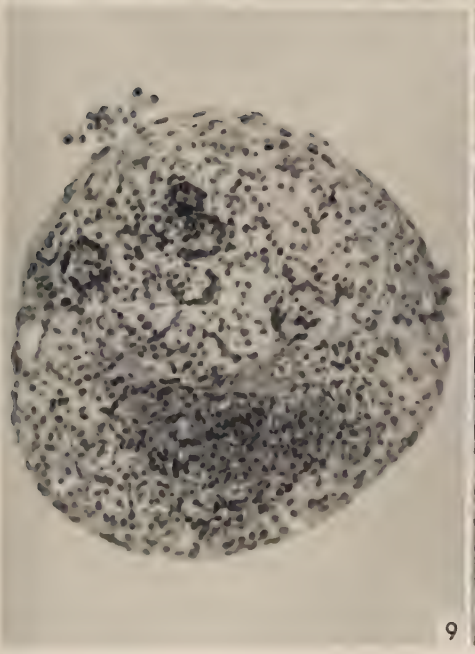

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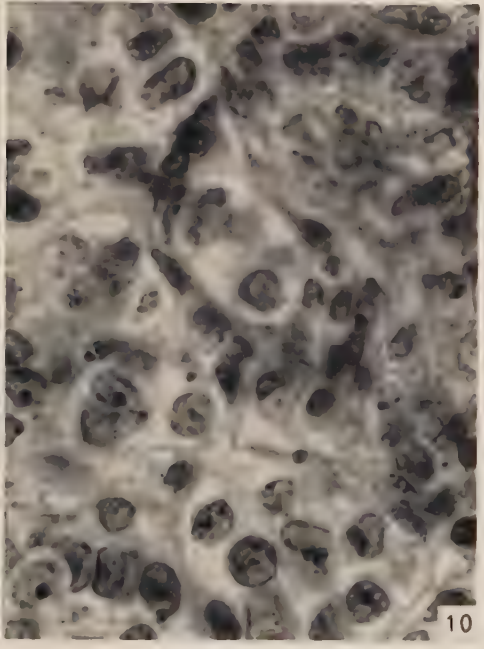

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also modified by the anterior hypophysis. At least under these conditions, therefore, the zona glomerulosa can be influenced by corticotrophic hormones, which agrees with the results of Tonutti [16].

In our previous experiments [14] somewhat contradictory results were obtained concerning the activity of the pituitary explants after a culture period of some days. If we combined fresh explants of the adrenal cortex with fresh explants of the hypophysis the morphological manifestations of extreme stimulation were still present after a combined culture period of twelve days. If we combined pituitary explants cultured for 9 days with fresh adrenal explants, manifestations of stimulation could still be observed. If, however, we combined pre-cultured adrenal cortical fragments with pre-cultured fragments of the anterior hypophysis, we noted only a minimal stimulation by anterior hypophysis that had been cultured for 6 days. This contradiction must have been due to our technique; it was later found that the functional activity of the hypophysis explants decreases rapidly if the transplantation from one medium to another takes too long. The present experiments have shown that the anterior hypophysis is still able to stimulate the adrenal cortex after a culture period of 12 days as indicated by the morphological changes produced *in vitro* and by the amount of corticosteroids present in the medium. This finding does not agree with those of Guillemin and Rosenberg [5] who found that, as determined by the Sayers test, no ACTH is produced by the hypophysis *in vitro* after a culture period of more than 4 days. Preliminary experiments in which we used the tissue culture technique of Guillemin and Rosenberg (roller tube technique as modified by Pomerat) confirmed these workers' results. If we cultured anterior hypophysis explants according to our "organ culture technique" we also found no activity as determined by the Sayers test after a culture period of 2 days. There are at least two ways of explanining this discrepancy, either the Saffran test is more sensitive than the Sayers test or we are dealing with two different mechanisms, one which decreases the vitamin C content of the adrenal cortex and which is only active for a short time after explantation, and one which stimulates the corticosteroid production and which is active for at least 12 days after explantation. Irrespective of which explanation may be right, our experiments prove that the anterior hypophysis is able to stimulate corticosteroid production without any influence of the hypothalamic centers.

SUMMARY

The influence of the anterior lobe of the hypophysis on the morphology and the function of the adrenal cortex *in vitro* has been studied. The explants were taken from 5-day-old rats. The "organ" culture technique as introduced by Gaillard was used.

Even after a culture period of 12 days the anterior hypophysis was still able to elicit typical morphological changes in the adrenal cortical cells and to stimulate corticosteroid production. This finding supports the results of other workers who found that the anterior hypophysis is able to produce corticotrophic substances without stimulation by the hypothalamus.

Both the cells adjacent to the capsule and those more centrally located in the adrenal cortex are influenced by the anterior hypophysis.

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Experimental Cell Research **15**, *484–495 (1958)*

THE EFFECTS OF ADENOSINE TRIPHOSPHATE AND DINITRO-*O*-CRESOL UPON THE FORM AND MOVEMENT OF *AMOEBA PROTEUS*.

A PRESSURE-TEMPERATURE STUDY[1]

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Since the well-known work of Mast [17] it has become more and more apparent that the cycle of sol ⇌ gel reactions, which can be observed so readily in any large free-living amoeba, must play an important role in the execution of amoeboid movement. But exactly how these sol-gel changes develop mechanical energy and precisely what metabolic reactions provide this energy are still questions which deserve more study.

A number of recent experiments (see Marsland [14]) have shown that pressure and temperature provide favorable approaches for an analysis of gelational phenomena in cells generally. Apparently the gelational state of the cytoplasm can be changed rapidly, reversibly and predictably under the agency of both of these variables. The formation of a gel structure in the cell seems to represent an endergonic process which likewise involves a positive volume change in the system [16]. Consequently the application of high pressure or low temperature tends to weaken the gel structures of the cell, i.e. to shift the sol ⇌ gel equilibrium (or steady state) in the sol direction.

In the amoeba, it has been shown [2, 15] that high pressure, in the range up to 8000 lbs/in^2, produces an exponential weakening in the structural state of the plasmagel layer and that the capacity of the amoeba to maintain its pseudopodia and to execute movement is correspondingly reduced. Moreover, the work of Landau, Zimmerman and Marsland [10] shows that low temperature likewise weakens the plasmagel system of the amoeba, and at lower temperatures the pressure levels required to suppress the formation of pseudopodia are significantly lower.

As to the energy requirements of protoplasmic sol-gel systems, considerable

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evidence now indicates that the high energy component of adenosine triphosphate may be involved. This is particularly apparent from the recent work of Ts'o, Bonner, Eggman and Vinograd [20], who studied the behavior of an ATP-sensitive protein (myxomyosin) extracted from an amoeboid slime mold; and from the work of Hoffman-Berling [6] and Weber [22], who studied the ATP-induced movements of various glycerol extracted cells. But also the work of Kriszat [7, 8] and of Runnström and Kriszat [18] would seem to point in a similar direction.

In the present experiments, accordingly, we have studied the effects of ATP and related compounds on the form and movement of *Amoeba proteus* under systematically varying conditions of pressure and temperature. Also we have studied the effects of 4,6-dinitro-*o*-cresol under similar conditions. This latter drug was chosen because it appears to act by interfering with oxidative phosphorylation reactions [12]. In each case the reagents to be tested were administered via the culture medium (Brandwein solution).

MATERIALS AND METHODS

Pressure-temperature equipment.—This has been described by Marsland [13]. The microscope-pressure chamber permits cells to be observed directly at magnifications up to 600 diameters while exposed to pressures up to 16,000 lbs/in^2. The pump allows for a pressure build-up of about 2000 lbs/in^2/sec; and the temperature housing, which is large enough to contain all glassware and solutions, as well as the microscope-pressure chamber, can be set at any point between $-5°$ and 60°C, with a maximum variation of ± 0.3°C.

Culture technique.—Vigorous mass cultures of *Amoeba proteus* were maintained in the dark at 18–20°C, in Brandwein [1] solution, buffered at pH 6.9. About 100 specimens, washed in fresh Brandwein solution and equilibrated to the proper temperature for 30 minutes, were used in each experiment.

Chemicals.—Adenosine triphosphate (ATP) was obtained from the Schwarz Laboratory, Mount Vernon, N.Y. The dry samples were placed in a dessicator and kept in a freezer. New samples of ATP were obtained every three weeks since some loss of activity was noticed after 4–5 weeks. Fresh solutions of ATP were prepared daily and kept in the refrigerator pending use.

Adenosine-5-monophosphate (AMP) was purchased from Bischoff Laboratories, Ivoryton, Conn., under the name of MY-B-DEN, in the form of ampules, each containing 20 mg AMP in 1 ml of distilled water. The 4,6-dinitro-*o*-cresol (DNC) was obtained from Eastman Kodak Co., Rochester, N.Y.

All solutions were made up in Brandwein and equilibrated to the experimental temperature before the specimens were added. The range of pH in the final immersion media was between 6.9 and 7.4. Control specimens observed in Brandwein solution buffered to the extremes of this range showed no appreciable change in form or movement.

EXPERIMENTS

ATP effects on amoeboid form and movement

Before studying the temperature and pressure parameters of ATP treated specimens, it was necessary to ascertain the effects of exposing the amoebae for various periods to a suitably chosen range of ATP concentrations. The object of these experiments was to find a concentration in which the animals would continue to display an essentially normal form and behavior, and thus to obtain favorable material for a pressure-temperature analysis of pseudopodial stability.

Amoebae exposed to a relatively high concentration (0.002 *M*) showed an immediate and drastic alteration of form and activity. Within 30 seconds locomotion ceased and then gradually the pseudopodia were retracted. After 20–30 minutes the characteristic picture was that of a dark rounded amoeba with a large central vacuole and with many small hyaline blebs radiating from the surface, see Kriszat [7]. Specimens left in this medium persisted in this motionless state until they died; but if within 24 hours they were thoroughly washed and transferred to fresh Brandwein solution, they became essentially normal in form and movement within 2–3 hours.

At an intermediate concentration (0.001 *M* ATP) only about 20 per cent of the specimens became rounded, blebbed and quiescent; but when the concentration was lowered to 0.0005 *M*, at least 95 per cent of the animals retained their normal amoeboid form and motility quite indefinitely. This then was the concentration chosen for the temperature-pressure experiments.

ATP effects on pseudopodial stability under various pressure-temperature conditions

Previously it was found [10]: (1) that the pseudopodia become unstable and the amoebae round up into motionless spheres when they are exposed to suitably high pressure; (2) that the pressure level required to induce this condition becomes less as the temperature is lowered; and (3) that the instability of the pseudopodia is related to a solation (loss of gel strength) in the plasmagel layer induced by the pressure-temperature conditions. Consequently, it seemed logical to use these pressure-temperature criteria for testing the effects of ATP upon *pseudopodial stability*.

In these experiments the amoebae (about 100 specimens) were placed in 0.0005 *M* ATP-Brandwein solution, previously equilibrated to the temperature of the particular experiment, and allowed to stand for 30 minutes in

the microscope-pressure chamber, pending the application of the test pressure. After the pressure was established, the amoebae were kept under observation for exactly 20 minutes. Then counts were made to determine the percentage of specimens that had lost all pseudopodia (fully rounded) as opposed to those that had retained a distinct vestige of their amoeboid form. Following the count, the animals were decompressed and it was observed that more than 90 per cent recovered an essentially normal form and behavior within about 20 minutes.

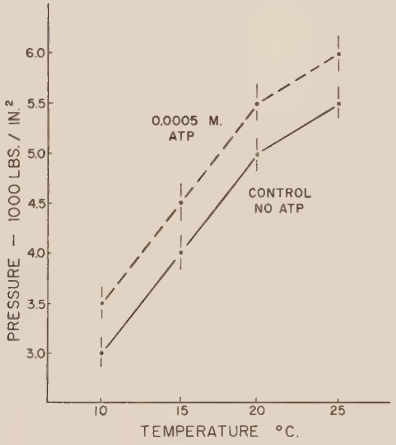

Fig. 1.—Effects of ATP upon the stability of pseudopodia in *Amoeba proteus*. Minimum pressures required to abolish all pseudopodia at various temperatures (10° to 25°C).

The results of these experiments are summarized in Fig. 1. At each different temperature (10°, 15°, 20°, and 25°C), a higher degree of pseudopodial stability was displayed by the ATP-treated specimens. In other words, at each temperature the pressure level required to induce a total loss of pseudopodial structure in 75 per cent of the specimens was consistently higher (by 500 lbs/in²) in the ATP-treated animals—as compared to untreated controls. This would indicate [10] that the plasmagel system of the amoeba maintains a firmer structure when low concentrations of ATP are administered by way of the external medium.

Effects of adenosine monophosphate and other phosphate compounds

Similar experiments were carried out using adenosine monophosphate (AMP), adenosine (Ad), dibasic sodium phosphate (Na_2HPO_4) and sodium pyrophosphate ($Na_4P_2O_7$). Here the object was to ascertain to what extent, if any, the high energy component may be involved in the mediation of the ATP effect. In these experiments, however, only one temperature (20°C) and

one pressure (5000 lbs/in^2) were used. Moreover, the criterion for determining the effects upon pseudopodial stability was the percentage of specimens that maintained pseudopodia, at least partially, under the specified pressure-temperature conditions (Fig. 2).

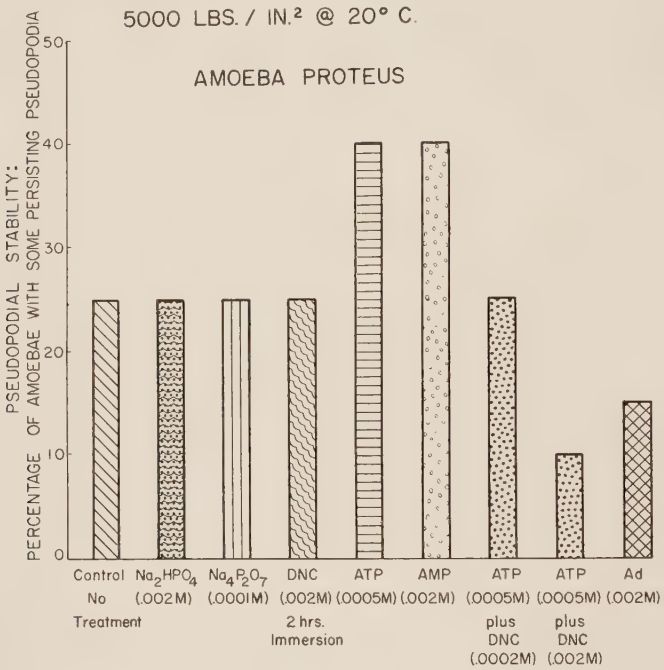

Fig. 2.—Effects of adenosine triphosphate (ATP), adenosine monophosphate (AMP), adenosine (Ad), inorganic phosphates (see above) and combinations ATP and DNC on pseudopodial stability in *Amoeba proteus*. In each experiment 100–150 amoebae were used and each value shows the percentage of non-rounded specimens, with some definitely persistent pseudopodia after a 30-minute exposure to high pressure (5000 lbs/in^2) at 20°C.

As might have been expected from a similar study on the cortical gel system and furrowing capacity of marine eggs [9], the only one of these compounds that tended to duplicate the action of ATP was AMP. With AMP, however, the required concentration was 4 times greater (Fig. 2).

Amoebae immersed in relatively strong AMP solutions (at and above 0.006 *M*) soon developed a dark, rounded, blebbed appearance, reminiscent of that of the ATP-treated specimens previously described; but in concentrations at and below 0.002 *M*, the animals remained normal in form and locomotion. Moreover, the specimens kept for 40 mins. in 0.002 *M* AMP and then

tested at 5000 lbs/in^2 (at 20°C) showed an enhanced pseudopodial stability equivalent to that of animals treated with 0.0005 *M* ATP (Fig. 2).

Sodium pyrophosphate, at 0.001 *M* concentration, gave marked signs of toxicity, including considerable cytolysis. However, in 0.0001 *M* solutions, $Na_4P_2O_7$ had little or no effect upon pseudopodial stability (Fig. 2). Adenosine (in concentrations up to 0.016 *M*) and Na_2HPO_4 (up to 0.002 *M*) did not induce any appreciable change in the form and locomotion and neither compound gave any increase in pseudopodial stability. Adenosine (Ad), in fact, made the pseudopodia distinctly less stable, as is shown in Fig. 2.

Effects of dinitrocresol (DNC)

This drug, in concentrations up to 0.002 *M*, had very little *immediate* effect upon form and motility; but very drastic changes ensued when the immersion periods were prolonged. In 0.002 *M* solutions the amoebae remained normal for about 2 hours. However, by the end of 6 hours all the specimens were completely rounded and inert, and many were definitely cytolyzed. In weaker solutions, namely 0.001 and 0.0001 *M*, the animals seemed normal for some 6 hours, but after 24 hours considerable rounding and quiescence had occurred. It would seem, therefore, that DNC may produce rather drastic effects, but that these are delayed, presumably by a slow rate of penetration into the cells.

Specimens tested within two hours of immersion in 0.002 *M* DNC, while they still appeared normal in form and activity, showed normal pseudopodial stability at 5000 lbs/in^2/20°C (Fig. 2). It was not possible to apply this test at later times, however, since too many of the specimens already had lost their pseudopodia and were rounded up.

Combination effects: ATP plus DNC

Even though DNC had no appreciable action upon form, movement or pseudopodial stability, except at relatively high concentrations and after long delay, it still seemed worthwhile to ascertain whether or not this drug could counteract the definitive effects of ATP, especially with reference to pseudopodial stability. Under the circumstances, therefore, it was somewhat surprising to find that DNC (0.002 *M*) did counteract the ATP effect even when the immersion period was as short as 30 mins. But more surprising, perhaps, it was also found that an overshoot occurred. In the presence of ATP, dinitrocresol, which alone gave no effect for periods exceeding 2 hours, now produced a marked decrease in pseudopodial stability within 30 minutes.

The results of these experiments, which were all performed at 20°C, are summarized in Table I. Here it may be noted that the capacity of the amoebae to maintain pseudopodia is considerably reduced at each of the different pressures used. The effect develops within 30 minutes and does not show much, if any, increase within the next 25 minutes. Moreover, the pressure differentials are quite large, namely 1500 lbs/in^2, when the ATP-DNC specimens are compared with normal controls, and 2000 lbs/in^2, when they are compared with specimens treated with ATP alone.

TABLE I. *Combined action of adenosine triphosphate (ATP) and dinitro-o-cresol (DNC) upon the pseudopodial stability in Amoeba proteus—in comparison with the action of each compound used singly.*

In each experiment, the percentage of completely rounded specimens, without any persisting pseudopodia, was determined after a standardized compression period of 20 minutes.

Treatment	Length of immersion (minutes)	Pressure (lbs/in^2)	Per cent rounded (no pseudopodia)
Controls (no treatment)	—	5000	75
ATP (alone) (0.0005 *M*)	30	5000	60
	30	5500	76
DNC (alone) (0.002 *M*)	55	5000	75
ATP (0.0005 *M*) plus DNC (0.002 *M*)	55	5000	90
	55	3500	76
	30	3500	74
	30	4000	87
	30	3000	69

Lower concentrations of DNC used in combination with ATP (0.0005 *M* standard) gave lesser effects. At a concentration of 0.0002 *M*, the stabilizing influence of ATP was exactly cancelled (Fig. 2); and at 0.00002 *M*, the ATP effect alone was found.

AMP plus DNC

Since it was found that AMP, in relatively high concentrations, tends to duplicate the ATP effects, it seemed possible that this compound might also have an influence upon the action of DNC upon the amoeba. This was not the case, however. Used in concentrations up to 0.002 *M*., AMP in the presence

of 0.002 *M* DNC had no appreciable effect upon the form, motility or pseudopodial stability, when tests were made during the first two hours of immersion.

DISCUSSION

These results, generally speaking, tend to support the concept that the gelated cortical cytoplasm (plasmagel layer) of the amoeba plays a dominant role, not only in maintaining amoeboid form and in stabilizing pseudopodial structure, but also in generating mechanical energy when amoeboid movement occurs. Moreover, the results suggest that the plasmagel is a contractile system which can receive energy from the metabolism of high energy phosphate compounds in the cell.

The recent work of Ts'o *et al.* [20] provides further support for the assumption that the plasmagel layer of the amoeba represents an ATP-sensitive contractile system [10]. These workers have extracted and partially purified an ATP-sensitive protein from the amoeboid slime mold, *Physarum polycephalum*, and have studied its viscosity and gelational behavior under a wide variety of conditions. Indeed, they have named this protein, myxomyosin, because of its resemblance to actomyosin [21]. It is of interest, therefore, to discuss the relationship of this work on cell extracts to the current work on whole cells.

Previous reports indicate that ATP may exert different effects upon a sol-gel system, depending upon various factors. The initial state of the system and the concentration employed seem to be very important, and also the method of application must be considered. Immersion and microinjection techniques may give different results and, of course, work with cell extracts represents another situation. It is necessary, therefore, to consider these factors, in attempting to relate the present results to those that have been reported previously.

The importance of initial state and concentration is very plainly indicated by the myxomyosin experiments of Ts'o *et al.* [20]. This work showed that adding ATP to the system when it is in a low viscosity state (which would seem equivalent to a sol condition) results in a marked but gradual build-up in the viscosity, or gelational state. If, on the other hand, ATP was added to a high viscosity (gelated) preparation, a very rapid and even more marked fall in viscosity (solation) occurred. Moreover, the reversibility of these changes was reduced when excess concentrations of ATP were employed.

All available evidence indicates that the formation of a gel structure in the protoplasm involves the bonding of certain protein components into a

three dimensional network and that the change from sol to gel condition is an endergonic process which involves a positive ($+\Delta V$) volume change. It seems reasonable to postulate, accordingly, that the gelation reaction receives energy from the hydrolysis of ATP. Indeed this postulate gains definitive support from the observation by Ts'o and collaborators [20] that the timing of the ATP-induced increase of viscosity, or gel state, in initially low viscosity myxomyosin preparations, is closely paralleled by the liberation of inorganic phosphate.

The contraction of a protoplasmic gel, which seems to involve a return to the sol condition, appears to represent a more problematical phenomenon. It seems possible that the protein components of the network are in a partially unfolded form and that the performance of work represents a forceful folding prior to a disassociation of the network. Or it may represent a forceful shifting of the protein micellae into a more compact network prior to the disassociation. In any event, it seems reasonable to postulate that ATP initiates the contraction of the gel network, when it is brought into proper spatial relationship within the cytoplasm. It seems possible, indeed, that contraction may involve the formation of an enzyme-substrate complex between the contractile protein and ATP. Thus following the contraction and disassociation of the network, energy for the reformation of the gel structure would be derived from the ATP system. And lastly, ATP resynthesized at some specialized locus in the cytoplasm, could be withheld from contact with the contractile protein, pending stimulation or the development of some other physiological condition which would call for a reactivation of the contractile system.

As reported by Goldacre and Lorch [5], by Goldacre [4] and by Ts'o *et al.* [20], the microinjection of ATP solutions into amoeboid cells results in a solation of the plasmagel in the region of injection, followed by a period of accelerated streaming. Under these circumstances a localized contraction of the plasmagel would be difficult to observe, although solation, which seems to be the inevitable aftermath of contraction, could readily be observed. Moreover, the accelerated streaming afterwards would seem to represent an enhanced fund of energy to support the sol-gel cycle rather than a decrease in the viscosity of the cytoplasm.

A comparison between results obtained by microinjection and those obtained by the immersion method brings up the question of permeability. A priori, it is difficult to understand how a large polar type of molecule, such as ATP, can penetrate into a cell from the surrounding medium. But it cannot be denied—on the basis of these and other experiments on amoebae and in view of our previously reported work on marine eggs [9]—that the

effects of ATP do penetrate into the interior parts of the cell. In the case of the amoeba, it might be postulated that ATP could be carried into the cell by the continuous infolding of the posterior parts of the cell membrane (described by Goldacre and Lorch [5]), which appears to be responsible for the absorption of neutral red; but such a mechanism could scarcely operate in marine eggs and other non amoeboid cells. It seems more feasible, therefore, to follow the suggestion of Lindberg [11] and Runnström and Kriszat [18] that ATP can transfer energy and/or labile phosphate across the cell surface to the subjacent layers of the cytoplasm and that the level of high potential phosphate energy within the cell can be raised by the presence of ATP in the outside medium.

In the present experiments, high concentrations of ATP in the external medium gave rise to generalized contraction and bleb formation, as was previously noted by Kriszat [7, 8]. Subsequently, moreover, the amoeba appeared to be abnormally stiffened and incapable of locomotion. But at a lower concentration (0.0005 *M*), which seems to provide for a more normal situation physiologically, the amoeba continued to display normal form and locomotion. However, the pseudopodia proved to be more stable when subjected to the solating effects of high pressure. This would seem to indicate that the gelation state was being sustained by an enhanced level of high potential energy in the cells.

The temperature effect (Fig. 1) in fortifying the gel structure of the pseudopodia, which has been reported previously [10], provides further evidence as to the endergonic nature of protoplasmic gelations. It is gratifying to note, therefore, that similar properties have been observed by Ts'o *et al.* [2] in their myxomyosin preparations. In the present connection, however, the point that deserves emphasis is that the fortifying action of ATP is superimposed upon that of temperature, within the range that has been studied.

The negative results obtained in the experiments with adenosine and with the inorganic phosphate compounds might indicate that none of these is limiting in the energy providing metabolic system, assuming of course that penetration occurs—as seems likely, at least with reference to the phosphates.

The positive results obtained with AMP—although it required a concentration some four times higher than that of ATP—would seem to indicate either that this compound, in the absence of a high energy constituent, cannot so readily transfer its effects into the cell; or that its effects within the cell are slower to develop. In any event, it seems most likely that AMP exerts its action by providing a precursor for the build-up of ATP. An interesting parallel is found, indeed, in the slow but steady, large increases in viscosity,

or gelational state, which were observed regularly by Ts'o and co-workers [20] in their AMP-treated preparations of myxomyosin.

The experiments with dinitrocresol alone (without ATP) are difficult to interpret. Ts'o and co-workers [20] reported that the closely related compound, dinitrophenol, employed at equivalent concentrations (0.001 *M*) stopped streaming in the slime mold within 6 minutes; whereas the amoebae, in our experiments, continued activity and showed little or no weakening of the pseudopodia for at least 2 hours. Perhaps the methyl derivative, dinitro-*o*-cresol, penetrates the cell more slowly, although Clowes [3] found this compound to be effective in blocking the cleavage of *Arbacia* eggs. Eventually, however, dinitrocresol did abolish amoeboid movement, presumably by interfering with oxidative phosphorylation [3, 12, 19].

The combined effects of DNC and ATP are also difficult to understand. Alone, DNC (0.002 *M*) gave no appreciable effect within 2 hours; but with ATP (0.0005 *M*), a marked weakening of the pseudopodia developed within 30 minutes. Possibly energy derived from ATP facilitates the transport of DNC across the cell surface. No doubt, the presence of DNC inside the cell would soon result in lowering the high energy phosphate levels; but at present we have very little evidence in reference to these questions.

SUMMARY

1. When a relatively high concentration (0.002 *M*) of adenosine triphosphate was added to the normal culture medium (Brandwein solution) the amoebae slowly contracted, each into a dark rounded mass with numerous surface blebs and a large light central area.

2. At a lower concentration (0.0005 *M*) the amoebae continued to display normal form and movement. However, the pseudopodia displayed an increased stability when subjected to the solating effects of high pressure.

3. The stabilizing effects of ATP on the pseudopodia was measured at four temperatures (10°, 15°, 20° and 25°C) and in each case the pressure differential amounted to approximately 500 lbs/in^2.

4. Similar positive results were obtained when adenosine monophosphate was substituted for ATP, but in this case the concentration required was about four times higher.

5. Essentially negative results were obtained using adenosine, inorganic phosphates (Na_2HPO_4 and $Na_4P_2O_7$) and dinitro-*o*-cresol.

6. Dinitro-*o*-cresol, which alone gave little or no effect (except after several

hours), produced a marked weakening of pseudopodial structure when used in combination with ATP.

7. These results and those of previous experiments are discussed in relationship to the hypothesis that the plasmagel of the amoeba is a contractile system which can receive energy from the metabolism of high potential phosphate compounds in the cell.

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A STUDY OF THE CELL NUCLEI OF THE ADRENAL CORTEX OF THE WHITE RAT BY MEANS OF INTERFERENCE MICROSPECTROGRAPHY

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Conflicting views and theories have been put forward concerning the histophysiology of the adrenal cortex. This can partly be explained by the variability of its architecture according to the animal species considered.

We owe the first histological description of this gland to J. Arnold [2] who already divided the cortex in three zones: glomerulosa, fascicularis and reticularis. It has often been attempted to correlate this morphological zonation with a functional one. Extensive investigation of the human adrenal of pathological cases brought N. Goormaghtigh [15] to the conclusion that the inner part of the zona fasciculata was the seat of greatest cell activity. He also suggested that a very thin juxta medullary layer was connected with androgenic activity. N. Hoerr [16] and H. Bennett [5] on the other hand attributed the function of elaboration and secretion to the outer part of the fascicularis. Thus H. Bennett divided the adrenal cortex of the cat into four functional layers: the presecretion, the secretion, the post-secretion and the senescent zone.

With the discovery of the corticosteroids and their differenciation in 17- and 11-ketosteroids it was necessary to examine if the different zones represented the seat of a different physiological activity. Some investigators believe that this is actually so.

Thus according to Sarasan [22], Deane and Greep [10], Deane, Shaw and Greep [11], Josimowitch, Ladman and Deane [17], the zona glomerulosa produces the mineralocorticoids and the zona fascicularis the glucocorticoids while, according to Allen and Slatter [1] the zona reticularis is responsible for the production of androgens.

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Mention must also be made of a theory, chiefly based on studies of adrenal regeneration following enucleation, which considers that the glomerulosa serves exclusively as a source of cells [9]. It should be remembered in this connection that already much earlier Goormaghtigh [14] had tried to prove that the glomerulosa was a reserve zone able to take over the function of cells of the deeper zones damaged in the course of pathological processes. Chester Jones and Wright [9] developed the theory of cell migration according to which a continual migration of the cells should take place towards the medulla. The cells nearest the medulla would become senescent –an opinion that had been already expressed in some earlier works. In the opinion of these authors the seat of elaboration of the hormones is located in the outer layer of the fasciculatis.

It is obvious that the problem would be solved by the direct histochemical detection of the active ketosteroids in the cells, but no specific reaction has been devised as yet. Lison [19] doubts if it ever will be possible to visualize the ketosteroids as they are present in the cell in extremely small amounts. On the other hand recent cytophysical techniques are able to assess accurately the functional activity of an individual cell and consequently can contribute to the solution of the problem which concerns us. We refer to the ultraviolet microspectrography, the microspectrophotometry in visible light, the roentgen absorption method [12, 18] and the interference spectrograph [23].

Barer already used a refractometric method for the evolution of the protein content of the cell by means of the phase contrast microscope [4]. Application of interference microspectrography makes possible the direct measurement of the optical path difference. It has been shown that the anhydrous mass m, per unit area in a homogeneous object situated in water is given by the formula $m = \varphi_\omega / \chi$, where φ_ω = optical path difference in water, $\chi = 100\ \alpha$, α being the specific refractive increment. The total dry weight of the object will be $m = \varphi_\omega \cdot A / \chi$.

For heterogeneous objects, as is always the case in cytology, φ_ω has to be integrated over the area $A = \int (\varphi_\omega \cdot A / \chi)\ dA$.

It has been demonstrated that the specific refractive increment of a protein in water is about 0.0019, while for dry proteins this value is 0.0015. In glycerin medium this value is about 0.09, but further experimental evidence is needed to verify this figure.

With the aid of the interference spectrographic method for the determination of the nuclear dry weight we have tried to obtain some information about the cell activity in the different layers of the adrenal cortex in various experimental conditions.

MATERIAL AND METHODS

White male rats were used for the experiments. The adrenal glands of three groups, each consisting of three animals, were investigated. A first group of animals served as controls, a second group received during 14 days a daily dose of 5 I.U. ACTH by intramuscular injection, the third group was treated during the same period with a daily dose of 5 mg hydrocortisone. After this period the animals were killed by a blow on the head. The adrenals were quickly removed, sectioned in two halves and immersed into liquid propane cooled down to 170°C by liquid air. From the propane they were transferred to the freeze-drying apparatus. The freeze-drying technique used was the one originally described by Glick and Malmström [13] and modified by Moberger, Lindström and Andersson [20].

The glands were embedded in paraffin, *in vacuo*. To isolate the nuclei of 4 different zones of the adrenal cortex paraffin sections were made of about 30–40 μ thickness. After removal of the paraffin, the sections were transferred to glycerin and brought under the phase contrast microscope. By means of the Fonbrunne micromanipulator, the zona glomerularis with the capsule, the outer, the inner layer of the fascicularis and the zona reticularis were dissected and the tissue fragments were transferred to separate quartz slides, and covered by a quartz coverslip, as the same preparation was used for interference microspectrography and ultraviolet spectrography. By slightly squashing and shearing of the coverslip, the cells were ruptured, and the nuclei which are much more resistant to mechanical injury than the cytoplasm, especially in the zona fascicularis, became isolated. All these manipulations were carried out in strictly water-free glycerin, the same day as the measurements. If this was impossible, the preparation was stored in a desiccator, *in vacuo*, in a deep-freezer.

In the present investigation, the determination of the optical path difference was performed with the scanning interference spectrograph described by G. Svensson. The measurements of φ were performed in waterfree glycerin medium. In every layer of each adrenal gland 20 nuclei were measured. Since the specific refraction increment α in glycerin is insufficiently known, we did not calculate the exact value of the anhydrous mass. Consequently all the results will be expressed as $m \cdot \chi$, the relative dry weight. χ being a constant, we are entitled to compare these values between each other.

For the statistical analysis of the data, parametric-free methods were used, as the populations are not normally distributed. For this reason we omitted the calculation of the standard deviation and the standard error.

RESULTS

Table I gives a survey of the mean values of $m \cdot \chi$, the relative dry weight, obtained in the different animals.

If we consider first the group of the control animals we observe some differences between the four layers. The highest value of the dry weight is found in the zona fascicularis externa. The dry weight of the nuclei in the zona glo-

merularis is somewhat lower, but the Kolmogorov–Smirnov test shows that this difference has only low significance ($0.207 > p > 0.10$). This test shows however that the difference between the zona fascicularis externa and interna is significant ($0.05 < p < 0.10$), and highly significant between the zona fascicularis externa and the zona reticularis ($p < 0.001$).

TABLE I.

Interference				
Controls	RK_4	RK_5	RK_6	Pool
Zona glomerularis	3.63	4.08	3.97	3.90
Zona fascicularis ext.	4.09	4.11	4.92	4.28
Zona fascicularis int.	3.66	3.58	3.62	3.62
Zona reticularis	2.79	3.10	3.90	3.31
ACTH	RK_{11}	RK_{12}	RK_{13}	Pool
Zona glomerularis	4.37	5.59	5.62	5.08
Zona fascicularis ext.	4.54	5.74	5.74	5.02
Zona fascicularis int.	4.93	5.01	6.05	5.27
Zona reticularis	3.97	5.36	6.96	5.40
Hydrocortisone	RK_{14}	RK_{15}	RK_{16}	Pool
Zona glomerularis	4.70	3.73	4.41	4.26
Zona fascicularis ext.	4.64	3.83	5.83	4.57
Zona fascicularis int.	3.85	3.87	4.44	4.05
Zona reticularis	4.47	3.52	4.72	4.17

In the control rats, the distribution of the nuclear anhydrous mass shows a gradient, decreasing from the zona fascicularis externa to the zona reticularis.

After administration of ACTH, the dry weight of the nuclei increases in in every layer. But statistical analysis shows that this increase is not significant in the zona fascicularis externa ($p > 0.3$). In the three other layers this increase is highly significant ($p < 0.001$). But when comparing the mean values of the nuclear dry weight in the four layers of this experimental group, we observe that they are increased in such a way that they tend to reach a same value. The median test shows more objectively this tendency: the probability that there exists a difference between these populations reaches only a value of $0.30 > p > 0.20$, which has only low significance.

After administration of hydrocortisone a similar reaction is noted in the

cortical nuclei: the relative dry weight of the nuclei tends to a same value in the four layers. The median test shows only a probability of $0.50 > p > 0.30$, which is not significant. The increase of the dry weight in the hydrocortisone group is however not so pronounced as in the ACTH group. This observation is confirmed by the Kolmogorov Smirnov test: comparison of every layer of the ACTH-stimulated adrenals, with the corresponding layer of the control group, shows a highly significant increase in the zona reticularis ($p < 0.001$); a low significant increase in the zona reticularis interna ($0.20 > p > 0.10$), and no significant increase in the zona fascicularis externa ($0.50 > p > 0.30$).

Table II.

	Volume ratio			Dry weight ratio			Concentration ratio		
	Control	ACTH	Hydrocortisone	Control	ACTH	Hydrocortisone	Control	ACTH	Hydrocortisone
Z. glomerul.	1	1.2	1.1	1.1	1.5	1.2	1.1	1.2	1.1
Z. fasc. ext.	1.8	2.2	1.1	1.2	1.5	1.3	0.7	0.7	1.2
Z. reticul.	1	1.4	1	1	1.6	1.2	1	1.2	1.2

When comparing these values with the values of the nuclear volume, determined in earlier experiments (H. Roels, 1956, unpublished), we get some information about the concentration of the nuclear material. Taking the mean value of the dry weight and volume in the zona reticularis as a base for our calculations, we compared the volume and dry weight ratios in the different layers. Dividing the dry weight ratio by the corresponding volume ratio, we obtained the concentration ratio in the different layers. Table II gives us a survey of these results. As we did not calculate the volume of the zona fascicularis interna, this layer is not considered.

In the control rats, nuclear concentration is highest in the zona glomerularis, somewhat lower in the zona reticularis and lowest in the zona fascicularis externa. ACTH treatment has nearly no influence on the nuclear concentration: a slight increase is observed in the zona glomerularis and zona reticularis. In the hydrocortisone group, the nuclear concentration is unaffected in the z. glomerularis, in the z. reticularis and a slight increse is noted, but in the zona fascicularis externa a marked increase is observed: nuclear concentration is nearly doubled.

DISCUSSION

By assessing the anhydrous mass of the cell nuclei in the adrenal cortex in two experimental conditions we have attempted to investigate the functional activity of the cortico adrenal cells and to investigate if a specific function can be attributed to the different cortical zones.

If we consider the values obtained in the group of the untreated animals, we observe some differences in the nuclear anhydrous mass between the four cortical zones: there exists a decreasing weight gradient from the zona fasciculatis externa to the zona reticularis. The dry weight of the zona glomerularis is the same as in the zona fascicularis externa.

After administration of ACTH, the dry weight increases in the cell nuclei, reaching a value which equals that of the zona fascicularis externa of the normal rat. In this layer, the nuclear anhydrous mass remains fairly constant. That the nuclear dry weight increases in the zona glomerularis after ACTH treatment, supports the opinion of some authors that ACTH exerts an influence on this layer [24]. Increase of the dry weight in the zona fascicularis interna and zona reticularis would indicate an increase of cell activity. The increase of nuclear dry weight after chronic stimulation of cell metabolism, has also been reported in the liver cell by Bahr [3].

Inhibition of the cell function by hydrocortisone, also results in an increase of the anhydrous mass of the nucleus. In the liver, an analogous observation has been made by Bahr [3]. In the adrenal cortex this increase does not reach very high values, and is only significant in the zona reticularis.

Comparing the values of nuclear anhydrous mass, with the volume, we have calculated the concentration ratios in the different layers. In the control rat, nuclear concentration is highest in the zona glomerularis, nearly the same in the zona reticularis and lowest in the zona fascicularis externa, where the dilution of the nuclear material is nearly twice this of the nuclei of the zona glomerularis.

Bahr showed in his work on the liver cell nucleus, that increased cell activity was accompanied by decrease of nuclear concentration. The high water content of the nuclei in the zona fascicularis externa would thus indicate a high functional activity of these cells. ACTH administration has practically no influence on the nuclear concentration. The dry weight increase is compensated by a proportional increase of the volume. No conclusion can be drawn from this type of experiment. More interesting are the hydrocortisone experiments in which the nuclear concentration is unaffected in the zona glomerularis, increased in the zona reticularis at the same ratio as the dry weight

increase, and particularly increased in the zona fascicularis externa. In this instance it is increased nearly twice.

This last result supports the hypothesis that we put forward earlier, that the zona fascicularis externa is the seat of normal glucocorticoid elaboration.

TABLE III.

	Zona glomerularis	Zona fasciculari externa	Zona fasciculari interna	Zona reticularis
Rat 4 control	8.30	12.71	9.93	8.24
Rat 11 ACTH	7.11	12.00	10.15	9.49
Rat 16 Hydrocortisone		8.61		

The large nucleoli in the zona fascicularis externa are also in favour of this hypothesis. During our measurements we were often able to trace a scanning line through the nucleolus. The phase difference was always higher than in the rest of the nucleus; but in the zona fascicularis externa of the normal and stimulated rats, this phase change was even so high that it surpassed the capacity of the instrument. In the zona reticularis of the normal rat and in the zona fascicularis externa of the hydrocortisone rats, the phase difference caused by the nucleolus was significantly lower. These observations indicate a higher concentration and accumulation of material in the nucleoli of the zona fascicularis externa of the control- and ACTH group than in the zona reticularis of the controls and zona fascicularis externa of the hydrocortisone group.

As mentioned earlier, measurements were also made by means of the ultraviolet spectrograph.

As the time spent at the Department of Cell Research of the Karolinska Institute was restricted, these experiments are still incomplete. Nevertheless we wish to mention them here, because their results fit in with our hypothesis.

Table III gives a survey of the preliminary results. The measurements were performed in one rat only in each experimental group, and in rat 16 only the zona fascicularis externa has been measured.

These values represent the mean of twenty corrected measurements $\varepsilon_{2.560}$–$\varepsilon_{3.150}$.

In the control rat the extinction shows the same decreasing gradient as the dry weight. This means that nucleic acid content is highest in the zona fascicularis externa. More experimental evidence is needed to establish the significance of the changes induced by stimulation and to exclude biological variations.

After hydrocortisone administration the nucleic acid content of the z. fascicularis externa is decreased. In earlier experiments we showed a decrease of 11 per cent of nuclear DNA in this layer following hydrocortisone treatment [21]. The decrease observed in the U.V. region exceeds this value to a large extent which indicates that this variation of nucleic acid content is mainly due to a decrease of nuclear RNA. From the work of Caspersson [7, 8] and Brachet [6] we know that nuclear RNA is correlated with cell function: the high RNA content of the nucleus in the zona fascicularis externa indicates an active cell function in this layer. The decrease of nuclear RNA in this layer after hydrocortisone treatment pleads in favour of the specific glucocorticoid function of these cells.

SUMMARY

By means of interference microspectrography the nuclear dry weight has been determined in the four layers of the adrenal cortex of a group of control rats, ACTH-stimulated and hydrocortisone-inhibited rats.

In the control rats a decreasing weight gradient is noted from the zona fascicularis externa towards the reticularis. After ACTH-treatment these values are increased in such a way that in every layer we find the same value. After hydrocortisone, we find also an increase but less pronounced.

By comparing these values with the nuclear volume the changes of nuclear concentration are discussed. This concentration is lowest in the zona fascicularis externa and highest in the zona glomerularis and reticularis in the control rats. ACTH-treatment has practically no influence on the nuclei of the adrenal cortex. After hydrocortisone treatment the nuclear concentration is increased in the zona fascicularis externa and also in the zona reticularis although less. The nuclear concentration in the zona glomerularis is unaffected.

These observations support the hypothesis that the zona fascicularis externa is the seat of normal glucocorticoid elaboration, while the deeper layer constitutes a reserve of less active cells, which may be stimulated in order to compensate higher needs of glucocorticoids.

Although these experiments do not allow a definitive conclusion about the

activity of the cells in the zona glomerularis, the high concentration of the material of these nuclei suggests a low cell activity.

Preliminary results of the measurements of the nuclear nucleic acid content by means of the UV spectrograph are given. They seem to corroborate the conclusion mentioned above.

We wish to express our feelings of deep gratitude to Professor Caspersson, for introducing us in this difficult field of cytophysical research.

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CYCLING IN THE OXYGEN CONSUMPTION OF CLEAVING EGGS

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In a series of papers Zeuthen [5–11] has reported that the dividing eggs of various echinoderms and the marine worm *Urechis* displayed waves in the oxygen consumption. Cytoplasmic cleavage in this material was, according to various interpretations, either correlated with a decreasing or with a minimal to increasing respiratory rate. These observations were based upon determinations in which several hundred eggs were sedimented in small Cartesian divers.

The information obtainable from mass runs is naturally limited by the degree to which the cleavages are synchronized. This pertains also to determinations on single eggs in the later divisions. After the fourth division it is no longer possible to observe accurately when cleavages occur in the diver, and to what extent later waves in the flotation pressure are mitotic in nature becomes increasingly uncertain. Therefore the first two or three divisions of a single egg provide the most reliable data.

In 1952 Scholander, Claff and Sveinsson [2] published a reference diver technique sensitive to 0.2 micro microliter (0.2×10^{-6} mm^3), which was capable of following the oxygen consumption in single large cells. The technique was applied to various protozoans and to single eggs of several of those species which had previously been studied in mass runs [3, 4]. The phenomenon of cycling was confirmed in general, but cleavage was found in all cases to take place associated with an acceleration of the oxygen consumption. In several species the cycling was found to be inconstant from one egg to another, and it could be altogether absent, as for instance in some eggs of *Urechis* and *Chaetopterus* [4, 1]. Cycling often diminished in amplitude after the first or second division. Zeuthen [9, 11] credited those single cell data which showed cycling as confirmation of his thesis that mitosis is constantly associated with cycling in respiration, but rejected the non-cycling data as inconclusive.

NOTES ON TECHNIQUES

Before attempting to interpret the significance of necessary cycling in the flotation pressure it is useful to scrutinize the limitations of the analytical methods used, and we shall therefore stress some sources of error in technique which have not been fully appreciated, or which remain unclear, namely, temperature control and the method for determining the flotation pressure.

If the respiration chamber has no water phase, fluctuations in temperature may, under ideal conditions, be compensated for by connecting the free end of the manometer with a moist compensating chamber submerged in the water bath. However, this will only work provided the rate of temperature adjustment is the same for both chambers, which is rarely the case, and it will of course not take care of the Q_{10} effect on the respiring material. The conditions are less favourable when respiration is run in water, for such a system becomes increasingly uncompensated the larger the water/gas ratio. The relations will be seen from the following easily derived equation:

$$U = 10{,}000 \left(\frac{T_2}{T_1} - \frac{G(T_0/T_1) + W\alpha_1}{G(T_0/T_2) + W\alpha_2} \right), \qquad (1)$$

where U = uncompensation in millimeters of water when the total pressure in the diver is 1 atm, T is the absolute temperature, α is the solubility coefficient, G is gas, and W is water volume.

Zeuthen [6] stated that pressure changes in the diver due to temperature were automatically adjusted by the use of a large compensating bottle. He therefore either let the bath temperature drift, or relied upon rapidly running sea water as temperature control. Measurements were made to test the constancy of the latter arrangement and revealed temperature fluctuations of 1°C (Fig. 1). The experiment was repeated on another day, picked at random, and similar results were obtained. Such an instability might well be expected, since the temperature of cold water running through a warm building fluctuates with the flow.

Temperature fluctuations of only 0.2°C will by the Q_{10} effect induce 2–3 per cent variations in the respiratory rate, and fluctuations of 1°C, like those in Fig. 1, will induce more than 10 per cent respiratory cycling. Added to this Q_{10} error will be the effect of temperature unbalance upon the measurement itself. In some *Psammechinus* runs [7], the liquid/gas ratio in the diver was 10/1, while in others, and also in the *Urechis* runs [8], it was between 2/1 and 3/1. It can be calculated from eq. 1 that the uncompensation in flotation pressure per 1°C change would amount to 43 and 11–16 mm of

water respectively, and from the reported rates of respiration (0.5–1 cm water/min) one may calculate that temperature fluctuations of only 0.1 to 0.2°C in the water bath would produce as much variation in flotation pressure as observed in most of the experimental runs which showed early cycling. It is hardly surprising that the *Urechis* runs, with a cycling of only 2–3 per cent gave conflicting timing of the cleavages. Blank runs, although valuable as

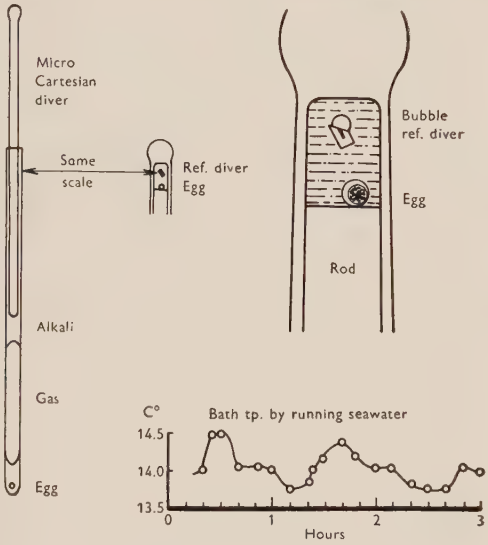

Fig. 1.—Micro Cartesian diver by Zeuthen for single cells (*left*). Reference diver (*right*), same drawn to scale of the micro Cartesian diver (*middle*). *Below:* temperature fluctuations in water bath run through by sea water at Friday Harbor, Wash., July 21, 1951 (Scholander and Sveinsson, unpublished).

indicators, are limited in that they are static, without a Q_{10} effect, and are not run at the same time as the respiratory determination. Claims that the cycling under the circumstances was significant to 1 per cent or better are clearly out of order, for even with the most scrupulous temperature control such a standard is very difficult to obtain.

Another important question of technique is how to determine best the flotation pressure. Since the gas pressure diminishes steadily in the diver there is never a static equilibration between the free and dissolved gas. Yet the accuracy of the technique depends upon the ratio between them remaining constant. Ideally this exists as long as the gas volume is kept constant, i.e., as long as the operator strives by continuous and even manipulation to keep the diver constantly at its mark, taking frequent readings on the manometer. Zeuthen, working with a relatively large and sluggish system advocates infrequent clocking of turning points as a better way of getting accurate timing. This undoubtedly would work in an all gas system, but when the water/gas ratio is high one may argue that diffusion lag in the system would result in

a poor definition of the free/dissolved gas when the diver turns. The hydrostatic level of the turn is a matter of chance, which introduces additional uncertainty in the effective flotation pressure, and the infrequency of reading, moreover, makes it impossible to estimate justly the scatter inherent in individual observations.

There are at the present time two techniques available which have been used with some success for respiratory studies in single cells, namely, the reference diver system already mentioned, and a micro Cartesian diver developed by Zeuthen [10]. The latter is essentially a micro edition of his earlier diver used for mass runs, provided with a new type of manometer burette, by which the flotation pressure can be changed by as little as 0.03 mm water. The sensitivity is given as 2–4 $\mu\mu$l.

In the micro Cartesian diver the egg is placed inside the diver and the whole system is brought to flotation. In the reference diver system a minute diver floats independently within a stationary respiration chamber containing the egg. Fig. 1 shows the size relation of the two systems. In both of them the sensitivity ultimately depends upon the smallest molar change in gas volume which will accelerate the diver. On this score the reference diver has a substantial advantage, in that it is several thousand times smaller than the micro Cartesian diver.

We shall present below data on the oxygen consumption during cell divisions in the eggs of *Psammechinus miliaris*. This egg was previously investigated in mass runs by Zeuthen, and was used by him as a test object in his recently developed micro diver.

EXPERIMENTAL

The sea urchins were secured near Bergen.

Eggs and sperm were obtained by injection of isotonic solution of KCl. The eggs were fertilized, and one egg was selected after the formation of the fertilization membrane. The jelly coat was stripped off by passing the egg repeatedly through the narrow opening of a pipette, after which the egg was cleaned by repeated washings in sterilized sea water.

The oxygen consumption was measured by the reference diver technique [2, 4], keeping the water bath temperature within 0.01°C. The following improvements of technique were introduced. The Saran filament, instead of being squashed flat to close the capillary, was touched at the end with a glass rod dipped in melted paraffine, which was drawn a few millimeters into the bore by capillary force. The filament was cut, leaving a plug of paraffine in the lower end of the diver (Fig. 2 *E*). The cutting is easy if the tip of a razor blade splinter is used as a knife and steadied on the support (Fig. 2 *E*).

RESULTS

The data from two typical experiments are presented in Fig. 2. Curves *B* and *D* are plotted as magnified deviations from the linear rate of oxygen consumption, according to a method described previously [4]. Curve *D* is

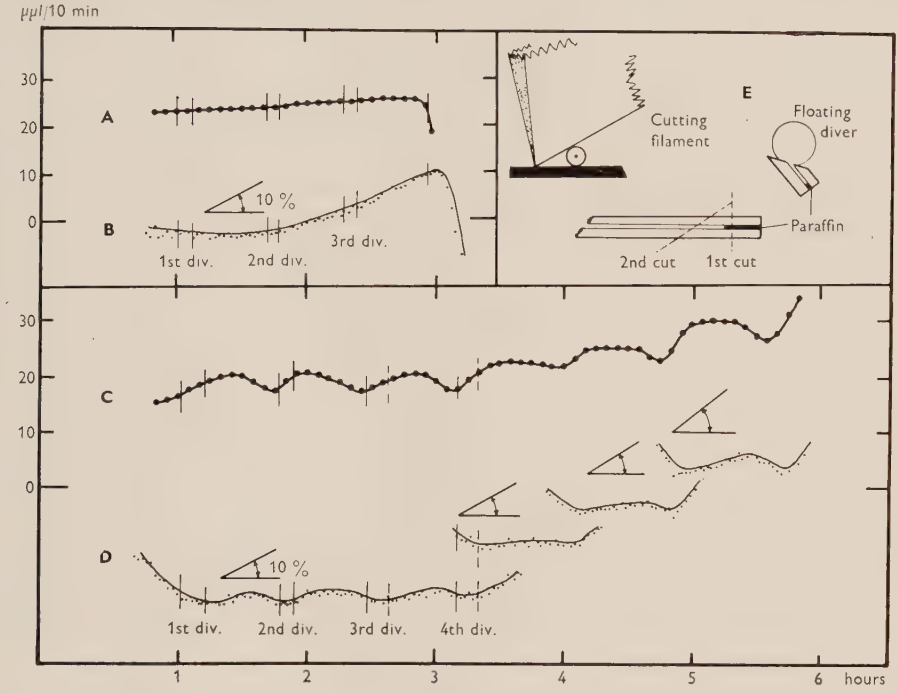

Fig. 2.—Respiration in single eggs of *Psammechinus miliaris* at 16.9°C. *A*, Rate curve constructed from *B*, which latter gives deviation from a straight line of cumulative readings, each point being one observation. *C*, Rate curve of another egg, constructed from *D*, which gives deviation from four straight lines fitted to the cumulative readings. The slope of 10 per cent deviation from straight line is indicated. *E*, Cutting of reference diver from 60 micron diameter Saran filament.

given as the deviations from four different straight lines matching the slopes of various parts of the curve. Above each section the slope of a 10 per cent change in rate is indicated. In Fig. 2, *A* and *C*, conventional rate curves have been drawn based on *B* and *D*. The rate is calculated from the slope of the tangent to the smooth curve drawn through the spread of observations in *B* and *D*. Each cell division as observed in the respiration chamber is marked on the curve with vertical lines. The starting point for each division can be

determined rather accurately, but the time of complete separation is less certain.

Examples of curves from two different eggs are given in Fig. 2. The egg represented by *C* and *D* showed a persistent cycling in oxygen uptake. During the first divisions the oxygen consumption fluctuated without any overall increase in rate. Later each wave resulted in a net increase in oxygen uptake. For the first four divisions the cytoplasmic cleavage could be observed in the chamber, and here it was always associated with an acceleration in the oxygen uptake. In the respiration a fifth, sixth, and seventh wave were apparent, possibly in association with cell cleavages, but this could not be observed. In contrast we have curves A and B from another egg, which divided without any significant wave in the oxygen uptake. Towards the end the curve drops precipitously as the oxygen in the water became exhausted.

CONCLUSIONS

As had been observed before in several other species of eggs, cycling of the oxygen consumption in *Psammechinus miliaris* is an inconstant phenomenon, occurring in some individual eggs and not in others. When it was present, we found as in earlier runs that the cytoplasmic division took place in an acceleration phase. Some of the runs were continued after the third or fourth division of the eggs, that is, when we could no longer observe the actual division time, and we could then see in some cases instances of late cycling. There were other cases, not illustrated, where cycling was not present in the beginning, but developed later.

The single cell studies published by Zeuthen on this same species [11] show cycling with the cytoplasmic division usually in the accelerated phase, which agrees with what we have consistently found in all species of eggs. Some of the rather considerable irregularities, both in regard to curve shape and time intervals, suggest an instrumental origin, and might be expected considering the relatively enormous diver.

Considering all available data from single cell runs, it is increasingly indicated that mitotic respiratory cycling is not a constant phenomenon. In the same batch one egg may cycle 10 per cent or more, another not at all. Cycling may increase or decrease after the first division or it may develop later. Significantly, however, one aspect seems regular and that is: whenever there is cycling the cytoplasmic cleavage always seems tied in with an acceleration in the oxygen consumption.

SUMMARY

Respiration has been measured in single cleaving eggs of *Psammechinus miliaris,* using the reference diver technique. It was found that cytoplasmic cleavage may take place without significant change in oxygen consumption, or that it may be associated with a transient acceleration in the oxygen uptake resulting in mitotic cycles. The results confirm previous single egg data obtained with the reference diver technique, and bring into focus events which evidently cannot be clearly observed with a flotation system several thousand times larger. Some limitations and shortcomings in published data on mass runs are discussed.

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FACTORS INFLUENCING SUBSTRATE UTILIZATION BY *TETRAHYMENA PYRIFORMIS*[1]

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Osmotic uptake of water by cells as a consequence of penetration by nutritional substrates has been claimed by Christensen [3] for ascites tumor and by Itoh and Schwartz [8] for rat-liver slices. Furthermore, Corbett [4, 5, 6, 7] has shown that the generally observed decrease in average cell volume of many bacteria and Protozoa with aging of cultures is associated with reduced rates of substrate-particle entry and consequent loss of water by the organisms. It was also shown that transfers from early logarithmic cultures to fresh medium resulted in initial contraction of cells, and those from maximum-density cultures produced initial cellular expansion, loss or gain of water depending on rates of substrate utilization.

In the present study, *Tetrahymena pyriformis* was employed to determine the factors which influence rates of utilization of several specific substrates and, consequently, contraction or expansion. This ciliate, which was included in the original experiments dealing with microorganisms [4] was chosen because of its numerous, well-defined nutritional requirements.

MATERIALS AND METHODS

Tetrahymena pyriformis H. was obtained through the courtesy of Dr. Richard P. Hall and maintained in the synthetic medium of Kidder and Dewey [9], stock cultures being carried in weekly transfers by means of an inoculating loop to 5 ml of medium. Incubation was carried out in a water bath at 28.5 ± 0.5°C.

Ciliates were counted in a Sedgwick-Rafter chamber (1 ml), using a Whipple ocular micrometer disc, and with addition of a drop of 5 per cent formalin to kill the organisms. Volumes were calculated from microscopic measurements of length and width of cells on slides prepared by admixture of approximately 5 volumes of culture or test suspension to 1 volume of 5 per cent formalin, 12 or more individuals being measured within 5 minutes of mixing to obtain an average for the sample.

[1] This study was supported by a grant from the National Institute of Arthritis and Infectious Diseases, Public Health Service, No. E-1567.

Average cell volume was calculated as a prolate spheroid, wherein a and b were the major and minor semiaxes, respectively. Then

$$V = \tfrac{4}{3}\pi a b^2.$$

Due to their slightly flattened shape, the organisms were found to have a minor semiaxis decreasing from the observed width/2 to approximately 0.138 (length + width). Hence, in calculating volumes, b was estimated in accordance with this observation.

Isolation of single ciliates for cultivation was accomplished by repeated sampling of an optimal dilution of the suspension with an inoculating loop of 1 mm diameter, each loopful being examined microscopically to obtain single cells.

RESULTS

Since the experiments involved measurement of *T. pyriformis*, which is rapidly motile, formalin-killed cells were observed. At the outset, comparisons were made between volumes of ciliates killed in this manner and controls immobilized either by tight application of coverslips or by chilling at 8°C for 12 hours. Table I records these data, which showed no detectable error in volume as a result of formalin treatment.

TABLE I. *Comparison of volumes of* T. pyriformis *based on measurement of ciliates with and without addition of formaldehyde.*

Days in culture	Average volume ($\times 10^{-6}$ mm^3)	
	+ CH_2O	CH_2O-free
1	7.30	6.90
3	8.81	8.97
7	9.96	10.10
17	7.20	6.89
20	2.02	2.04

Average volumes of ciliates were determined on cultures of different ages. Fig. 1 presents composite data from numerous cultures. Observations were made also of volume changes in cells transferred from cultures of various ages to fresh medium. Table II records the initial cell volumes and those observed after an interval in fresh medium. It is evident that average cell volume was maximum at 4 to 6 days in culture, decreasing thereafter. In transfers from cultures of 2 to 15 days in age, ciliates underwent initial contraction, those from older cultures showing initial expansion.

Actual gain or loss of water in contraction or expansion was ascertained

by comparing loss on drying at 90°C of ciliates taken directly from early and late cultures with that of the respective cells after an interval in fresh medium. Table III shows that 16.5 mg of cells from a 4-day culture decreased to 11.5 mg within 1 hour in fresh medium, losing only 11 mg on drying, whereas 7.9 mg cells from a 16-day culture increased to 14 mg in fresh medium within 14 hours and lost 13.4 mg on drying. Hence, ciliates lost water in contraction and gained it during expansion.

TABLE II. *Volume changes after transfer to fresh medium.*

Days in culture	Cell volumes ($mm^3 \times 10^{-6}$) Initial	After transfer		
2	12.1	80 mins.	6.6	loss
4	13.6	80	7.7	
6	10.9	35	9.6	
6	11.5	25	7.9	
6	10.2	15	8.5	
11	9.7	53	8.1	
13	8.6	17	4.8	
15	6.0	30	3.2	
26	4.1	13 hrs.	6.0	gain
27	5.1	16	10.0	

TABLE III.

Days in culture	Wt. cells transferred (mg)	Hrs. in fresh medium	Wt. in fresh medium (mg)	Wt loss (90 °C, 2 hrs.)
4	16.5	1	11.5	11.0
16	7.9	14	14.0	13.4

Ciliates from a 21-day culture were observed for rates of initial expansion in medium at different pH levels. Also, those from a 27-day culture were transferred in different numbers to fresh medium to determine the possible influence of suspension density on the rate of expansion. Fig. 2 shows an optimum of pH 6 for expansion, and Fig. 3 shows more rapid expansion in suspensions of approximately 40,000 cells per ml than in less dense suspensions.

Experiments were carried out to detect a possible relationship between the lag in expansion of *Tetrahymena* in less dense suspensions and specific substrates by comparing the rate in medium with increased concentration of one nutrilite with that of controls in unaltered medium. The results, which are recorded in Table IV, show that yeast nucleic acid gave rise to equal expansion rates in suspensions of high and low population when this substrate was

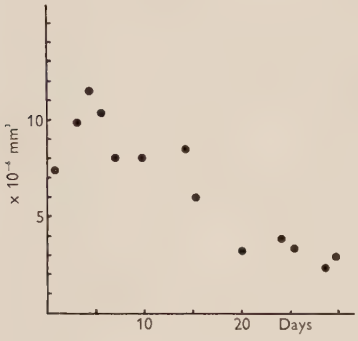

Fig. 1

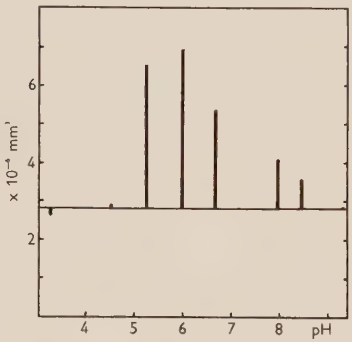

Fig. 2

Fig. 1.—Average volumes of ciliates in cultures of various ages.

Fig. 2.—Influence of pH on rate of initial expansion of ciliates after transfer from a 21-day culture to fresh medium.

increased from 0.1 to 0.4 mg per ml. On the other hand, L-leucine promoted expansion exceeding that of controls, and maltose increased expansion only slightly, presumably indicating that these two substrates were not related directly to the expansion lag in sparse populations.

Since the foregoing data suggested a requirement for conditioning of the medium by the ciliates prior to utilization of yeast nucleic acid, a further experiment was performed to confirm the identity of the substrate involved. It might be expected that any such demand on inoculated cells would be a limiting factor in relation to the maximum volume of medium in which a single cell can survive and reproduce. If enzyme production and/or action were involved in the conditioning, increased concentrations of the specific substrate might increase the maximum ratio of medium tolerated per cell by the law of mass action. Accordingly, single ciliates from a 27-day culture were placed in 5 ml volumes of medium in two series of 19 tubes each, one series containing 0.1 mg and the other 0.5 mg of yeast nucleic acid per ml. After 20 days incubation, the former series contained no successful cultures, whereas, with the higher substrate concentration, 6 cultures resulted.

Experiments were also carried out to study the relationship of initial contraction to substrate utilization. At the outset, rates of contraction of ciliates in fresh medium were compared with those in a solution wherein the amino acids of the medium were replaced by equimolar sucrose. The data of Table V are typical of the results, indicating that cellular contraction in fresh medium occurred at a slower rate than that in an isotonic solution of non-penetrating solute.

TABLE IV. *Influence of elevated concentrations of specific substrates on the lag in expansion of ciliates in suspensions of lower population.*

N = cells per ml.

		Ciliate volumes ($mm^3 \times 10^{-6}$)		
			14–16 hrs	
Substrate	mg/ml	Initial	Log N = 3+	Log N = 4+
L-Arginine	0.6	5.1	6.3	10.0
	2.4	5.1	5.0	10.1
L-Histidine	0.15	3.3	2.6	4.2
	0.3	3.3	2.3	
	0.6	3.3	1.7	
L-Leucine	0.07	4.1	3.0	6.0
	0.14	4.1	7.3	
	0.21	4.1	5.5	
	0.28	4.1	3.1	
L-Lysine	0.15	3.1	3.1	4.8
	0.3	3.1	2.7	5.6
	0.6	3.1	3.8	5.1
Maltose	2.5	3.2	4.0	6.0
	5.0	3.2	4.9	
	10.0	3.2	4.7	
Yeast nucleic acid	0.1	4.1	3.0	6.0
	0.2	4.1	4.5	
	0.3	4.1	5.1	
	0.4	4.1	6.3	
	0.1	3.9	3.2	4.1
	0.4	3.9	3.9	3.7
	0.1	5.8	6.0	9.5
	0.4	5.8	9.1	8.8

The effect of pH on rate of initial contraction was observed, ciliates having been transferred from a 4-day culture. The data of Fig. 4 show that contraction occurred most slowly at pH 6.7 and more rapidly at pH 8.5, 4.6, and 3.3. Also, comparisons were made of contraction rates of cells from 3 and 9-day cultures transferred to fresh medium in different populations. Fig. 5 records the results, which indicated that ciliates from the same culture con-

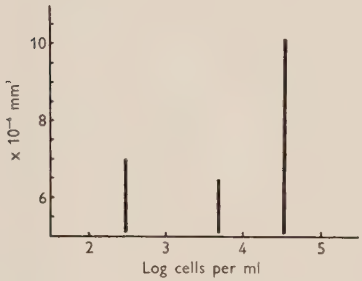

Fig. 3

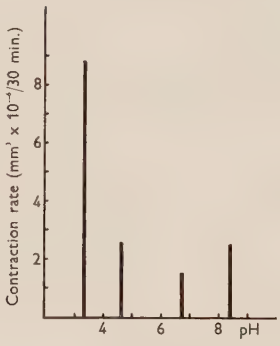

Fig. 4

Fig. 3.—Influence of cellular population on rate of initial expansion. Volumes of ciliates 16 hours after transfer from a 27-day culture to fresh medium are plotted against logarithms of ciliates per ml.

Fig. 4.—Influence of pH on rate of initial contraction of ciliates in fresh medium.

tracted as rapidly in higher populations as in lower ones. It should be pointed out that the curves for contraction of ciliates from the 3-day culture are more variable in slope due to their dependence on one volume measurement each but, nevertheless, show that no retardation of contraction occurred in the higher population. Cells from the 9-day culture continued to contract for longer times with lower populations. These findings suggested that contraction *per se* was independent of cell population but that its duration depended on

TABLE V. *Comparison of contraction rate in fresh medium with that in isotonic sucrose solution.*

Age of culture (days)	Ciliate volumes ($mm^3 \times 10^{-6}$)		
	Initial	Sucrose solution	Medium
10	7.8	5.3 (2 min.)	7.4 (10 min.)
13	9.8	7.4 (10 min.)	8.9 (18 min.)

attainment of conditions promoting expansion, which was influenced by population, as expected from previous data.

The retardation of initial contraction of *Tetrahymena* in the presence of growth promoting solutes was studied further by observing transfers to me-

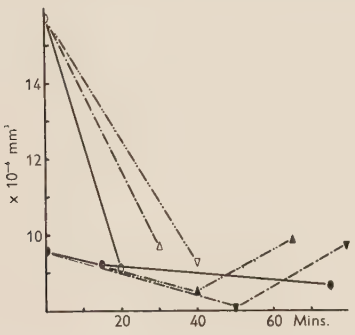

Fig. 5

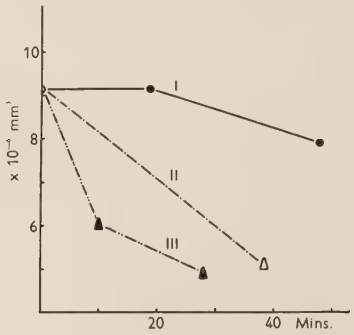

Fig. 6

Fig. 5.—Contraction rates of ciliates at different population levels in fresh medium. Open and closed symbols indicate transfers from 3 and 9-day cultures respectively, a circle, upright triangle, and inverted triangle designating progressively fewer organisms. Suspension densities from the 3-day culture were 12,800, 1280, and 128 per ml, those from the 9-day culture being 364,000, 36,400, and 1320 per ml. Cell volumes are plotted against time.

Fig. 6.—Contraction of ciliates from a 3-day culture in complete (I), L-arginine-free, and DL-serine-free (II and III) medium. Cell volumes are plotted against time.

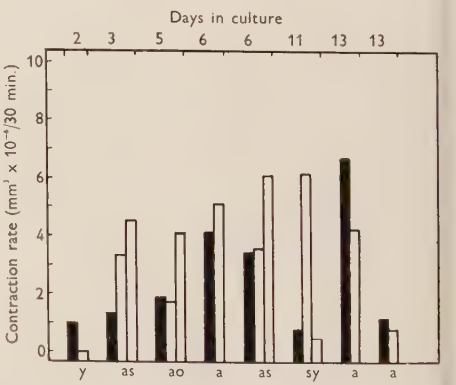

Fig. 7.—Loss in average ciliate-volumes per 30 minutes after transfers from cultures of different ages to complete-medium controls (solid bars) and deficient medium. Substrates omitted were yeast nucleic acid (*y*), L-arginine (*a*), and DL-serine (*s*). Ornithine replaced arginine (*o*) in one observation, giving acceleration of contraction.

dium lacking different specific substrates. Fig. 6 presents data from a typical experiment with ciliates from a 3-day culture, wherein the cells contracted more rapidly in medium lacking L-arginine or DL-serine than in controls of complete medium. Fig. 7 records results from these experiments with ciliates from cultures of different ages, showing that the effectiveness of different

nutrilites in retarding contraction varied with age of the parent culture. L-Arginine retarded contraction in ciliates from 3 and 6-day cultures and accelerated it in those from a 13-day culture. It is noteworthy that yeast nucleic acid did not retard contraction in any of the experiments which included it.

DISCUSSION

Preliminary comparisons of average cell volumes of *T. pyriformis* with and without addition of formalin indicated no appreciable volume alteration by the reagent within the five minutes required to complete an observation. This finding was in agreement with results of Bahr *et al.* [1], which, however, did show appreciable increases in tissue volume at 30 minutes.

T. pyriformis resembled *Euglena gracilis* [4] in undergoing a reduction of average cell volume with aging of cultures (Fig. 1). Transfers of ciliates from cultures of 2 to 15 days in age to fresh medium resulted in initial cellular contraction, those from 26- and 27-day cultures resulting in initial expansion. These findings were similar to those for *E. gracilis* and several bacterial strains previously studied [4] but were unique in that *T. pyriformis* contracted in transfers from cultures of a more prolonged age range and was, therefore, more suitable for study of the mechanism of initial contraction. That the volume changes observed in transfers of *T. pyriformis* were indeed associated with gain or loss of intracellular water similar to that of other organisms [4–7] was evident from the actual water contents of ciliates before and after contraction or expansion (Table III). A similar relationship of ciliate contraction and expansion to the rate of substrate uptake was indicated by the similar effect of pH variation (Figs. 2 and 4).

A dependence of initial expansion on population of *T. pyriformis* was evident in the present experiments (Fig. 3). With the expectation that increased substrate concentration would result in increased availability [2], within limits, if retarded expansion were due to sub-optimal activity of the specific enzyme, expansion rates were compared between optimal and suboptimal populations in unaltered medium and those in medium with increased amounts of different required substrates (Table IV). Yeast nucleic acid was the only substrate so tested which specifically eliminated the lag in expansion within the population range observed. Increased concentration of this substance also made possible the survival and growth of cultures when single ciliates were transferred to 5 ml medium. These findings support a hypothesis that expansion and growth of *T. pyriformis* in this medium depends upon a cellular conditioning related specifically to utilization of yeast nuecleic

acid. This action may consist of the secretion of a diffusible enzyme or, possibly, the alteration of a physical property of the medium. It is evident that the finite ability of a ciliate to effect such a change may be critical relative to the maximum volume of medium per cell permitting survival and multiplication.

Initial contraction occurred more slowly in the complete fresh medium than in controls wherein the amino acids were replaced by isotonic sucrose (Table V), suggesting that solute uptake continued at a progressively diminishing rate in the complete medium. This conclusion was suggested also by the finding that contraction rate depended on pH (Fig. 4), and that contraction was more rapid in transfers from a 3-day culture in the absence of L-arginine, and in transfers from 3, 6, and 11-day cultures in DL-serine-free medium (Figs. 6 and 7). The immediate availability of such substrates to transferred ciliates in fresh medium suggested that their uptake was independent of substances secreted by the cells, a hypothesis that received support from the finding (Fig. 5) that initial contraction-rate was not accelerated in lower populations.

The variation in effect of L-arginine from contraction-inhibiting to contraction-promoting with age of culture (Fig. 7) may reflect a variation in rate of cell growth between mitoses similar to that reported by Prescott [11] for *Amoeba proteus*. On the other hand, such a shift might conceivably result from a selective uptake of substrates which are growth-limiting at a particular phase in the parent culture. According to the latter view, free amino acids in the cytoplasm of *Tetrahymena* [12] may be in physicochemical equilibrium with those combined in the cytoplasm and, also, with those available from the medium. Such a system might include carrier molecules in the membrane similar to those postulated by Christensen [3] for ascites tumor cells.

That comparable variations in substrate uptake may occur in cells of metazoan tissues appears likely from data such as those of Paul and Pearson [10], wherein renewal of the medium resulted in reduced respiration of chick embryonic heart explants. Hence, such physiological variations require evaluation relative to seeming departures from the classical laws of osmotic behavior by cells.

SUMMARY

T. pyriformis H resembled other Protozoa and bacteria in the occurrence of reduced average cell volume with aging of cultures, and in undergoing initial contraction or expansion in transfers from cultures of comparable growth stages to fresh medium.

Initial expansion of the ciliate depended upon substrate uptake and consequent increase in intracellular water. Such cellular expansion was achieved through a conditioning of the extracellular medium by the organism, which was prerequisite to utilization of yeast nucleic acid and related to the number of ciliates present. The same conditioning was found to be critical relative to the maximum volume of medium per ciliate permitting growth.

Ciliates undergoing initial contraction continued to take up certain substrates, including L-arginine and DL-serine, at progressively diminishing rates after transfer, indicating that these substances did not require any diffusible secretion by the cells for their uptake. Thus, the collective data suggest that the uptake of at least one substrate by *Tetrahymena* depended on conditioning of the medium, that of certain amino acids being independent of any such conditioning.

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THE EFFECT OF 200 R OF X-RAYS ON THE RATE OF SPERMATOGENESIS AND SPERMIOGENESIS IN THE MOUSE

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After an injection of adenine-8-^{14}C into male mice, DNA in spermatogonia and spermatocytes is initially labelled and the tracer is retained in the differentiating spermatogenic cells up to the stage of ejaculated spermatozoa [14]. The progress of spermatogenesis, and the intervals between various spermatogenic stages and ejaculation, can be estimated from these data and from the timing of the repeatable association of cell types in the seminiferous epithelium [8, 15, 16]. Some of the previous estimates were based on males which received a very low dose of irradiation; in the present experiment, the males injected with tracer were irradiated with 200 r of X-rays, a dose of practical interest to the radiation geneticist. Spermatogenesis in normal and irradiated males was then compared by estimating the percentage of labelled spermatozoa ejaculated at various intervals after the injection of adenine. Significant differences between control and irradiated males in the interval between the injection of tracer and the ejaculation of labelled spermatozoa would indicate changes in the rate of spermatogenesis following irradiation. Data will be presented to show that irradiation had no obvious effects on the progress of spermatogenesis.

MATERIALS AND METHODS

Five outbred male mice, aged 1.5 months and of average weight 28 g, were each given an intraperitoneal injection of 1.2 μc adenine-8-^{14}C (specific activity 4.6 mc/mM) per gram of body weight. Three days later three of the males were irradiated with 200 r of X-rays to the testes while under Nembutal anaesthesia. The two other males were not irradiated and served as controls. The constants of the X-ray machine were: 140 kVp, 220 r/minute, inherent filtration $\cong$ 1 mm Al., plus 1 mm Al. filter, H.V.L. $\cong$ 3.2 mm Al. Each male was mated at approximately 7, 14, and 21 days after

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the injection of tracer (i.e. 7, 14, and 21 days p.i.) and the ejaculated spermatozoa rejected. Each male was then mated at intervals of 2–3 days from 25 until 41 days p.i. and thereafter at intervals of 4–5 days until 66 days p.i. when the experiment was terminated. The females used were superovulated with gonadotrophins [2, 12] in order to control the time of mating. As from 25 days p.i., spermatozoa were recovered from the uterus immediately after mating, their density of numbers estimated under the microscope, and then made into smear preparations for autoradiography. They were coated with Kodak AR 10 film and exposed for 40 or 48 days.

In every ejaculate at least 200 well isolated spermatozoa were scored for the presence of an autoradiograph, except for five samples in which, owing to scarcity of spermatozoa, only 100 were scored. Labelled spermatozoa can produce either definite or weak autoradiographs according to the degree of labelling, and both types were considered as positively labelled. In preparations with the greatest percentages of labelling, spermatozoa with weak autoradiographs reached up to 20 per cent of the number with definite autoradiographs. Weak autoradiographs were not due to the intrinsic background of the film, for they were absent from ejaculates containing no definitely labelled spermatozoa. To facilitate comparisons between males the percentages of labelled spermatozoa in the ejaculates of each male were expressed as the percentage of the maximum percentage attained by the male. Percentages given are for a standard exposure of 40 days, those obtained after 48 days exposure being reduced by an empirical factor of 0.5 per cent per day (total reduction 4 per cent). Only morphologically normal spermatozoa were scored, for atypical spermatozoa will probably not be capable of fertilizing eggs. However, striking differences in the number of atypical spermatozoa were not obviously apparent between irradiated and control males.

RESULTS

Both irradiated and control males mated after each pairing with females, except for male B at 25 days p.i. No evidence of oligospermia was found in the control males. One of the irradiated males (*D*) showed only slight indications of oligospermia, another (*C*) was oligospermic from 38–54 days p.i. (i.e. 35–51 days after the irradiation), and the third male (*A*) showed pronounced oligospermia between 32–54 days p.i. The method of examination for oligospermia, by microscopical examination of the density of spermatozoa in the ejaculates immediately after mating, is sufficiently sensitive to detect pronounced changes in the numbers of ejaculated spermatozoa, though mild degrees of oligospermia might not have been detected. Bateman [1] recorded oligospermia between 35 and 49 days after 200 r of X-rays to mice.

One control and two irradiated males ejaculated a low percentage of labelled spermatozoa at 30 days p.i., and all males ejaculated many labelled spermatozoa at 32 days p.i. This agrees with results from other non-irradiated males [14, 15, 17] where higher percentages at 30 days p.i. are due to exposures of the film longer than used here. Irradiation had therefore not delayed

the appearance of the first labelled spermatozoa in the ejaculate. The percentage of labelled spermatozoa increased after 32 days p.i. in all males, the maximum percentages and the time of sampling being given in Table I. High percentages of labelled spermatozoa were ejaculated for some days by all males. However, the percentage of labelled spermatozoa from irradiated male *A* was lower than expected, and male *C* reached its maximum earlier than normal, both of these events possibly being due to the pronounced oligospermia after irradiation.

TABLE I. *Actual maximum percentages of labelled spermatozoa in ejaculates of irradiated and control males.*

Male	Days after injection of tracer	Percentage of labelled spermatozoa
Irradiated		
EM2A	41	45.0[a]
EM2C	35	72.0[a]
EM2D	45	79.3[b]
Control		
EM2B	41	64.5[b]
EM2E	45	88.5[b]

[a] Exposure of the film: 40 days.
[b] Exposure of the film: 48 days, converted to 40 days after subtraction of 4 per cent.

The percentages of labelled spermatozoa in the ejaculates of each male, expressed as percentages of the maximum attained by that male, are plotted in Fig. 1. The increase in percentages of labelled spermatozoa, and the period when the percentages were high, were similar in control and irradiated males. After 45 days p.i. the percentage of labelled spermatozoa in the ejaculates decreased, but irradiated males ejaculated higher percentages than control males.

DISCUSSION

Irradiation can interfere with the labelling of DNA (see [3, 10]), but sufficient cells were labelled by three days after the injection of the tracer to follow the rate of spermatogenesis after irradiation. The most mature spermatogenic stage which initially incorporates tracer into DNA is probably the resting spermatocyte [15, 17], and the most mature stage labelled by the

time of the irradiation would therefore be three days older, i.e. zygotene. This is calculated as follows. The interval between the latest resting spermatocyte stage and earliest pachytene is three days [8]. However, it is very improbable that the synthesis of DNA will proceed until the latest part of the resting spermatocyte stage, and hence zygotene is indicated as the most mature stage labelled at irradiation. (These intervals correspond to the average

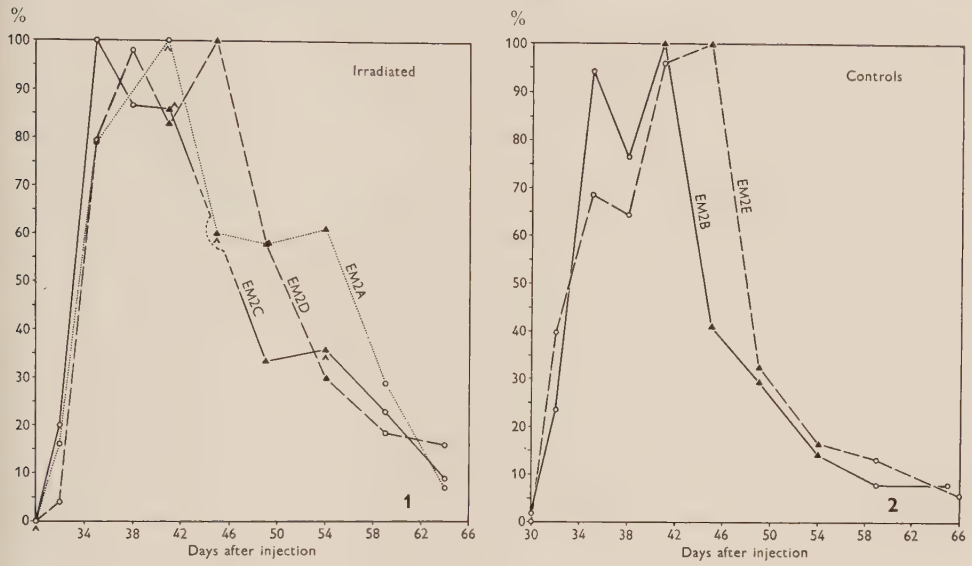

Fig. 1.—The percentage of labelled spermatozoa in ejaculates of irradiated and control males expressed as a percentage of the maximum attained by each male [1, 2, 3].

Actual percentages obtained by exposing the film: O for 48 days, ▲ for 48 days and converting to 40 days by subtraction of 4 per cent.

Spermatozoa of all males showed no positive autoradiographs at 25 and 28 days p.i. (male EM2B was not sampled at 25 days p.i.) except for spermatozoa of male EM2D which showed 3.5 per cent positive autoradiographs at 28 days p.i.

For each point 200 sperm-heads were scored, except for points marked ∧, for which 100 sperm-heads were scored (at 30 days p.i. this mark applies only to male EM2A).

rate of spermatogenesis [16, 17]). Labelled spermatozoa ejaculated at or just after 30 days p.i. were therefore irradiated at zygotene, and those ejaculated later were irradiated at earlier stages of spermatogenesis. The fact that the ejaculation of labelled spermatozoa commenced at the same time in control and irradiated males shows that the irradiation had caused no delay in spermatogenesis between zygotene and ejaculation. This also implies that meiosis was not delayed by damage to the chromsomes or spindle fibres, and agrees

with the results of Oakberg [6] and Whiting [19] showing that irradiation does not delay meiosis in mice and in female *Habrobracon*. The similarity in the rate of increase in the percentages of labelled spermatozoa in control and irradiated males after 30 days p.i. shows that no delay was caused between stages earlier than zygotene and ejaculation. In general, the similarity of the pattern of labelling in ejaculated spermatozoa of irradiated and control males indicates that the same average intervals occurred between the various spermatogenic stages and between these stages and ejaculation.

200 r of X-rays destroys almost all spermatogonia *B* [5]. After irradiation most spermatogonia *B* would not differentiate further and their progeny would be absent from ejaculates 35 days later (i.e. 27.6 days between latest spermatogonia *B* and release of spermatozoa [8]) plus 7.5 days transit down the vasa [15]. The males were irradiated three days after the injection of tracer, consequently the oligospermia should be apparent 38 days after the injection of tracer; this was evident in the results. The maximum percentage of labelled spermatozoa from irradiated male *A* was 45 per cent, which is lower than usually expected. This male had pronounced oligospermia from 32–54 days p.i., i.e. when high percentages of labelled spermatozoa are normally ejaculated. The low percentage from this male is presumably due to the destruction of many spermatogonia which incorporate tracer for some days after injection [11] and normally become mixed with unlabelled products of earlier and later generations [16, 17]; it is not indicative of a delayed ejaculation of spermatozoa through a retardation of spermatogenesis. Irradiated male *C* showed a maximum percentage of labelled spermatozoa earlier than controls; this may have resulted from a suppression of the normal peak caused also by the destruction of labelled spermatogonia.

The percentages of labelled spermatozoa decreased in both control and irradiated males after 45 days p.i., but were higher in the irradiated males. After irradiation many of these spermatozoa originate from the repopulating seminiferous epithelium. If the repopulation by dormant spermatogonia starts immediately after irradiation, the descendant cells should on average reach the ejaculate from 45 days p.i. onwards (i.e. 3 days between injection of tracer and irradiation, plus 34.5 days for the complete spermatogonial cycle, plus 7.5 days transit down the vasa). The higher percentages in irradiated males may then be due to some delay in the onset of repopulation by labelled spermatogonia after irradiation. The selective destruction by irradiation of dormant spermatogonia with unlabelled DNA might also increase the percentage of labelled spermatozoa in irradiated males. Irradiation as used here tends to destroy those cells in the first one-third to one-half of the

interphase, while cells in the second half, during which DNA is synthesized, are relatively resistant [3, 10]. Spermatogonia are dormant (in interphase) for 4–5 days [6]. If the timing of the cycle in other cells [3, 10] applies here, then irradiation three days after injection of tracer will tend to kill cells in the first part of the dormancy, which are labelled only in RNA [9], thus increasing above control values the percentage of DNA-labelled survivors from the second part of the dormancy.

The results show that after 200 r, (*a*) spermatogenesis proceeds at the normal speed, (*b*) there is no damage to the vasa at least as far as the progression of spermatozoa is concerned. The destruction of spermatogonia, resulting in the oligospermia at approximately 38 days p.i., would have lowered any pressure exerted by developing cells in the testis and epididymis on spermatozoa in the vasa. The absence of delay in the ejaculation of labelled spermatozoa therefore also shows that (*c*) for the progression of spermatozoa there is no *vis a tergo* from the seminiferous epithelium or the epididymis. These conclusions confirm and extend the results of Shaver [13] and Oakberg [6, 8] who found no delay between several stages of spermatogenesis after 100 r of X-rays, of Macmillan [4] who showed that no *vis a tergo* existed in the epididymis of the rat, and of Spalding *et al.* [18] who found that spermatozoa in the epididymis of the rat were normal 48 hours after massive doses of gamma rays. In control males the maximum percentage of labelled spermatozoa does not reach 100 per cent (for discussion see [17]), and therefore the present conclusions are based on the assumption that spermatogenic cells were destroyed by irradiation irrespective of whether or not they were already labelled in DNA, except for dormant spermatogonia; except for dormant spermatogonia the duration of the cell cycle of each spermatogenic stage [8] is such that, provided tracer is available, all cells capable of initial labelling would have labelled DNA by three days after injection. It is then assumed that at any spermatogenic stage radiation sensitivity is not determined by a different class of DNA metabolism i.e. a different ability to incorporate tracer in some cells of the stage. Differences in sensitivity within each spermatogenic stage are, as mentioned above, probably related to the cell cycle [7, 10].

SUMMARY

200 r of X-rays to the testes of mice previously injected with adenine-8-^{14}C does not delay the first appearance of or the increase in numbers of labelled spermatozoa in the ejaculate. From this evidence it is concluded that after this dose, (*a*) spermatogenesis proceeds at the normal speed, (*b*) there is no

damage to the vasa, at least as far as the progression of spermatozoa is concerned, and (*c*) for the progression of spermatozoa there is no *vis a tergo* from the seminiferous epithelium or the epididymis.

We are indebted to Professor C. H. Waddington, F.R.S., and to Dr. R. A. Beatty for their interest, and one of us (R. G. E.) to the Agricultural Research Council for financial support.

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ALPHA IRRADIATION OF PARTS OF SINGLE CELLS IN TISSUE CULTURES

I. TECHNIQUES

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One method of investigating the mechanism of the biological action of ionizing radiations is to irradiate a part of a cell which is in mitosis, watch it for a period by phase contrast, and subsequently fix it and stain it. Techniques for irradiating parts of cells have been reviewed recently by Ulrich [11] and Zirkle [13]. Several workers have used polonium alpha particles for work of this kind. Polonium^{210} has a half life of 138 days, and emits particles which have a uniform energy of 5.3 MeV. This energy gives them a well-defined range, the value of which depends upon the density and composition of the material through which they pass. In air at 15°C and 760 mm pressure, the mean range is 3.842 cm [5]. In water at 25°C it is 39.9 microns [1]; the range in soft tissue of unit density is similar to that in water. Since this range has the same order of magnitude as the length of many kinds of living cells, it is possible to irradiate the outer part of a cell by placing a polonium source at an appropriate distance from it, and, by employing cells which have nuclei in a convenient position, to arrange to irradiate part of the cytoplasm of a cell without irradiating its nucleus.

For example, Petrova [9] irradiated an alga which has cylindrical cells with a diameter of 35 μ and a central nucleus. The penetration of the alpha particles was controlled by interposing thin foils between the polonium source and the cells, and she showed that much higher doses were needed to kill the cells if the radiation was confined to part of the cytoplasm, than if the nuclei were included in the region which was irradiated.

The method used here to irradiate part of a cell has been to make a very small polonium source, and place it at a measured distance from a cell in a hanging drop preparation by means of a micromanipulator. The volume irradiated by one of these sources is limited by the range of the alpha particles, and it is placed in a position such that this volume includes the region of the cell which is to be irradiated.

Small sources have been made by depositing polonium on the tips of tungsten micro-needles. The polonium is covered by a thin layer of electrodeposited chromium, to seal it in position. The particles lose some of their energy in passing through the chromium, and emerge with a range in water

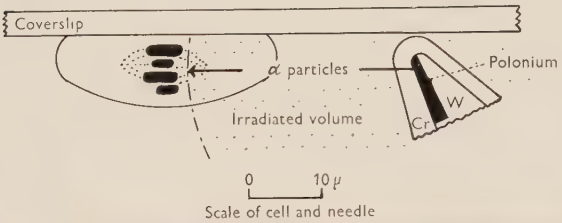

Fig. 1.—Use of a micro-needle carrying polonium to irradiate part of a chick fibroblast at metaphase.

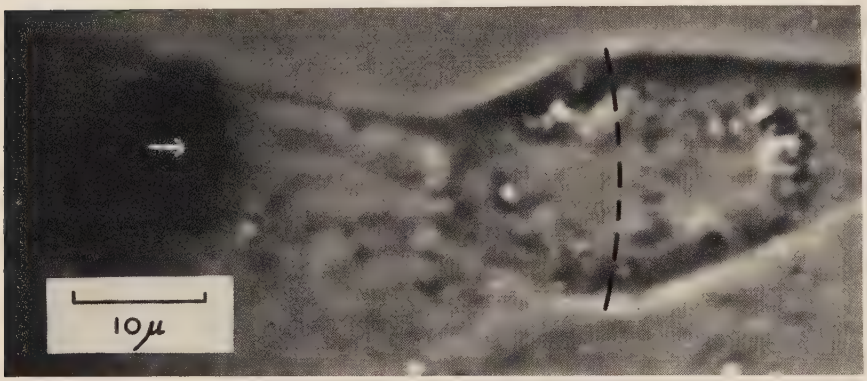

Fig. 2.—Micro-needle (marked with arrow) irradiating part of chick fibroblast at metaphase. The region between the micro-needle and the dotted line is being irradiated.

of between 20 and 35 microns, depending upon the thickness of the chromium, which varies from needle to needle. If one of these micro-needles is placed at a suitable distance from a cell in a tissue culture, a part of it can be irradiated; for instance, part of the cytoplasm and one half of the spindle, without the chromosomes, in a chick fibroblast at metaphase (Figs. 1 and 2). The cell can be watched by phase contrast during irradiation, and the presence of the micro-needle has very little effect on contrast and definition, even when an oil immersion objective is used.

The procedure in experiments with the micro-needles is, briefly, as follows. A tissue culture is mounted over medicinal paraffin on a "Chambre à huile" [2], and placed on the stage of a microscope, which is kept at 37°C. A cell which is approaching the stage which is to be irradiated is found in the outgrowth. The micro-needle carrying the polonium is guided into position, and

left at the required distance from the cell for a measured period, usually one minute. Time lapse photographs on 16 mm film are taken during and after irradiation, and usually before as well.

The techniques for tissue culture, micromanipulation, and for making the micro-needles are described in this paper. Subsequent papers will describe the technique for measuring how the dose rate of alpha radiation varies with the distance from the micro-needle, and the results obtained when parts of dividing chick heart fibroblasts have been irradiated. The methods and some of the results have been described briefly elsewhere [7].

METHODS

Tissue Culture and Micromanipulation

Cells used for micromanipulation have to be grown in a liquid medium, as a clot would obstruct the movements of a micro-needle. The method used here is a modification of the "Horseshoe" technique of Dr. H. B. Fell, described by Walker and Yates [12]. A drop of chicken plasma and a drop

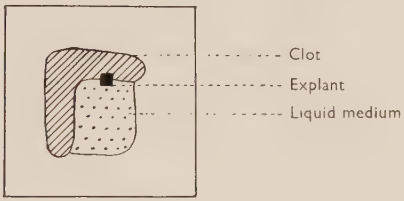

Fig. 3.—Tissue culture technique, providing cells growing in a liquid medium.

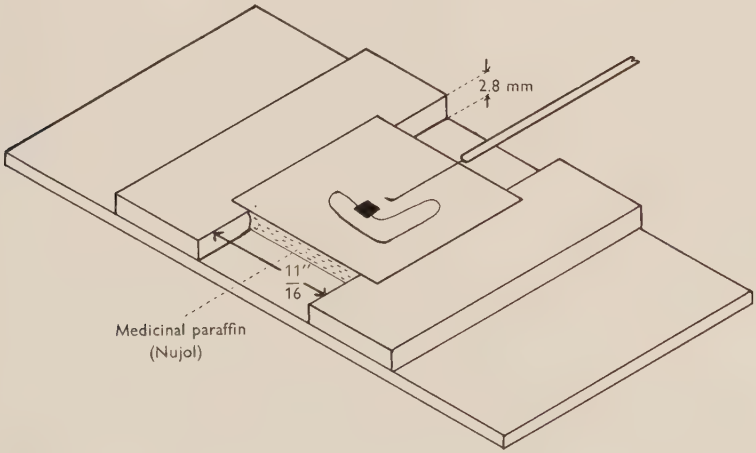

Fig. 4.—A tissue culture mounted over paraffin on a "Chambre à huile" for micromanipulation.

of chick embryo extract (1 part pulp from a 10-day embryo; 1 part Tyrode solution) are placed on a $\frac{7}{8}''$ square No. 2 coverslip, and mixed into a drop the shape of an inverted L. The explant, a fragment from the heart of a 7-day chick embryo, is placed near the upper arm of the L, and the drop is spread with a cataract knife, so that it just covers the explant (Fig. 3). When

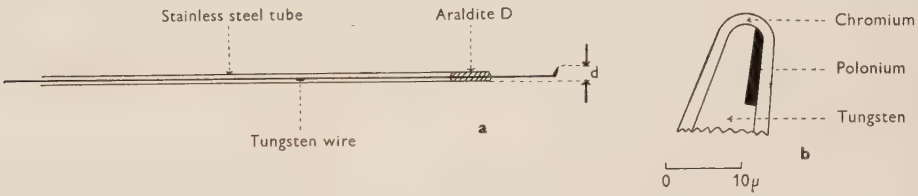

Fig. 5.—*a*, Mounted micro-needle; *b*, its tip, to a larger scale.

it has clotted, a thin layer of the liquid medium (the exudate produced when equal volumes of chicken plasma and embryo extract are mixed, allowed to clot, and the clot is cut into fragments) is spread over the region contained by the L. The culture is then inverted, mounted over a chambre à huile (Fig. 4) made by sticking two pieces of glass to a $3'' \times 1\frac{1}{2}''$ microscope slide with "Araldite type I Natural", and incubated at 37°C.

When the chamber is incubated, some cells grow out into clot, some into the liquid. The latter are used. The stage of the microscope is kept at 37°C by a warm box; the de Fonbrune micromanipulator enters this through a slit in its back. A Cooke long working distance phase contrast condenser is employed.

Micro-needles

The object of the techniques described below is to make tungsten micro-needles with a tip diameter of 3–5 microns, mounted in steel tubes in the manner shown in Fig. 5*a*, to deposit polonium on a length of about 10 microns of the outer side of the tip of a needle (Fig. 5*b*), and to seal this in position with an electro-deposited layer of chromium.

Mounting and sharpening the micro-needles.—A 3″ length of tungsten wire, 0.005″ in diameter, is passed through a piece of stainless steel tubing 1 mm in outside diameter, and sealed in at one end by a plug of "Araldite D". The wire projecting from the plugged end of the tube is cut to a length of 8 mm, and reduced to a fine point by electrolysis in 2*N* sodium hydroxide solution [10]. Twelve volts A.C. is applied, and the other electrode is a length of stout copper wire. The point produced by this A.C. electrolysis is usually

too fine and fragile, and so it is rounded off to a tip with diameter of 3–5 microns by electrolysis in the same solution with 12 volts D.C. The needle is made the anode. The voltages and the concentration of the solution are not critical, but it should be made up in distilled water with sodium hydroxide of analytical grade, and must be changed frequently.

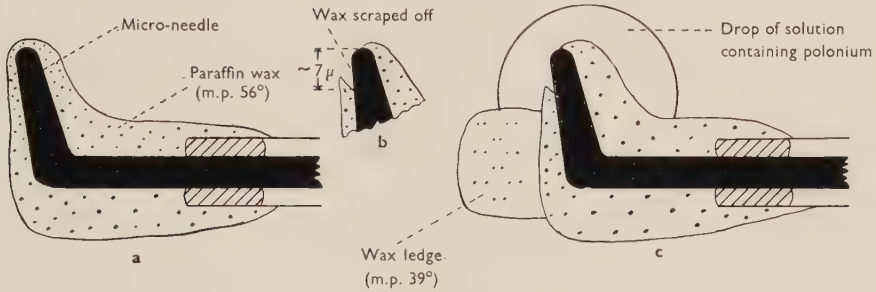

Fig. 6.—Stages in the application of polonium to a needle.

The wire is bent at a distance of 1–1.5 mm from the point, through an angle such that the outer edge of its tip is almost perpendicular to the steel tube, as in Fig. 5*b*. It is important that the depth of the needle (*d* in Fig. 5*a*) should be less than 2 mm, to allow it to be inserted into a chambre à huile. The wire must project a few millimetres from the tube, as shown in Fig. 5*a*, to ensure that, when it is placed near a cell, the steel tube does not obstruct the annular cone of illumination from the phase contrast condenser, and so impair visibility.

Applying the polonium.—The first step in depositing polonium upon one side of the tip of a needle (Fig. 5*b*) is to etch the tungsten by immersing it in boiling 20 volumes hydrogen peroxide solution for 30 seconds. Polonium deposits readily upon this etched surface from a solution in 0.5 *N* nitric acid. After etching, the needle is examined under the microscope. If the point has become too fine, or if the outer edge of the tip does not make the correct angle with the tube, as in Fig. 5*b*, the electrolysis and the etching process are repeated.

Next, the whole end of the needle is coated with paraffin wax. The wax is scraped off for a distance of 5–10 microns at the tip, exposing the part of the needle upon which polonium is to be deposited (Fig. 6*b*). A drop of a solution containing polonium is then placed upon the end of the needle (Fig. 6*c*), and left there for the polonium to deposit. The remainder of the wax is removed, leaving the needle ready for electro-plating with chromium.

The wax coat is applied by dipping the needle several times into filtered paraffin wax (m.p. 56°C) held just above its melting point. The integrity of the coating is tested electrically, making use of the potential difference of about 0.6 V that is developed between zinc and tungsten when they are immersed in normal sodium hydroxide solution.

To scrape off the wax at the tip of the needle, it is clipped to a thick perspex microscope slide and placed on the stage of a microscope. The wax is scraped off for a distance of 5–10 microns with a "micro-chisel", consisting of a fragment broken from the middle (the sharpest part) of a safety razor blade, fixed to a metal rod with black wax, and manoeuvred by the micromanipulator. The cut into the wax must be made at an angle, as shown in Fig. 6*b*; otherwise the wax does not come off cleanly.

Some more paraffin wax (m.p. 39°) is then applied to the needle so as to form a ledge (Fig. 6*c*). A drop of a solution of polonium in 0.5 *N* nitric acid, containing 1 millicurie/ml, is placed on it, and left for a few hours for the polonium to deposit upon the exposed tungsten. During this period, the needle is kept in an atmosphere which is saturated with water vapour, to prevent evaporation. The stainless steel tube in which the needle is mounted is covered with a sleeve made of PVC tubing, to protect it from contamination with polonium.

The polonium solution is removed with a fine pipette, and the needle is rinsed in 0.5 *N* nitric acid. The wax is removed by dipping it into very hot molten wax, and then soaking it in xylene for an hour. The hot wax is contained in an aluminium bottle-cap; a fresh one is used for each needle, to avoid transfer of polonium.

For examining the micro-needles at all stages in the process, a combination of a 12 mm achromatic objective and a ×15 eyepiece fitted with a graticule gives adequate magnification for measurement of their tips, and a sufficiently long working distance.

Chromium plating.—As a plating material, chromium has the virtue of hardness, and in control experiments with needles which had no polonium deposited upon them, has never given any suggestion of toxicity. Its greatest advantage is that the plating solution, which contains chromic acid, cleans off small fragments of dirt and wax which are often, unavoidably, left adhering to the tip of the needle when plating begins. On the other hand, it is deposited in a state of stress and the plating invariably breaks if a needle is bent. Cracks are always present, and it is not easy to produce a non-porous coating.

The general principles of chromium plating are set out in a number of works [3, 4, 6]. I deposit two layers from different solutions. The first contains 250 grams of Analar chromic acid, 2.5 grams of Analar sulphuric acid, and about 15 grams of iron per litre, and is made with distilled water. The second solution has the same composition, except that the iron is omitted. The first solution yields a softer, more adherent black coat [8]; the second adds a hard, white layer. In 25–50 per cent of the needles, the polonium is sufficiently well covered for it not to dissolve off when immersed in the serum-embryo extract culture medium. It may still come off in acid or alkaline solutions.

The apparatus is shown in Fig. 7. The plating bath is a 5 ml beaker; the anode is a coarse grid of platinum wire, 20 × 5 mm. An extra cathode, made from copper strip, is fixed about 3 mm behind the micro-needle. It projects below the needle; when the assembly carrying the electrodes and the needle is lowered into the plating solution, it ensures that the deposition of chromium begins as soon as the needle touches the surface of the plating solution.

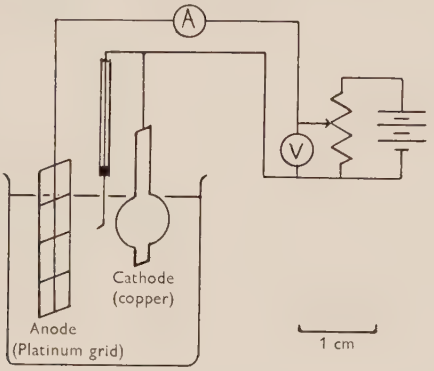

Fig. 7.—Apparatus for chromium plating micro-needles.

Immediately before a needle is plated, it is examined under a microscope. The surface near its tip is often covered by a layer of a blue substance, probably an oxide of tungsten, produced under the intense alpha particle bombardment. Chromium does not plate out on this; accordingly, it is removed by immersing the needle in boiling 20 volumes hydrogen peroxide for 20–30 seconds. Surprisingly, this violent treatment usually leaves plenty of polonium behind on the needle.

Then it is plated. In the first solution, the aim is to deposit a coat 2–5 microns in thickness. A current of 0.4–0.5 amps is passed. After a few seconds, the needle is withdrawn from the solution and examined under the microscope. Plating is continued until the coat has reached the required thickness. The same current is used in the second solution; here, 10–20 seconds usually deposits 1–2 microns of chromium. The current is supplied by four dry cells, to avoid the possibility of polonium being removed from the needle by the small reverse current present in rectified A.C. A fresh lot of the first solution is used for each needle, to ensure that, if polonium does dissolve in the plating solution, it will not be deposited on another needle.

After plating, the needles are rinsed with water, and immersed for five minutes in 0.1 *N* sodium carbonate solution, followed by two changes of distilled water. They are then ready for the chromium coating to be tested, and for the flux of alpha particles around them to be measured.

SUMMARY

Techniques are described for (1) growing chick heart fibroblasts under conditions which permit single cells to be subjected to micromanipulation; (2) making tungsten micro-needles; (3) depositing polonium upon their tips, and sealing this in position by an electro-deposited layer of chromium.

The micro-needles serve as sources of alpha particles which have a well-defined, measurable range of 20 -35 microns, depending upon the thickness of the chromium. They can be used to irradiate parts of single cells in tissue cultures.

I should like to express my gratitude to Dr. F. G. Spear, of the Strangeways Laboratory, Cambridge, who suggested that I should attempt this kind of work, to the Prophit Trust for a Studentship, the British Empire Cancer Campaign for a Junior Fellowship, and to the Saltwell Research Fund for a Scholarship. And I should like to thank Dr. Edith Paterson for her interest.

I am grateful to Professor G. de Hevesy for leave to publish this work in detail, and to Messrs. Oliver and Boyd for permission to include Fig. 1.

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II. DOSIMETRY

In the first paper of this series, techniques were described for making tungsten micro-needles with polonium deposited over a length of 5–10 microns of their tips, and for covering this polonium with a layer of chromium 2–6 microns thick [5]. Because of the sharply defined range of the alpha particles emitted by the polonium, these micro-needles could be used to irradiate parts of single cells in tissue cultures.

This paper describes a method for measuring the dose rate of alpha radiation at points near one of these micro-needles, when it is immersed in a liquid tissue culture medium (in this series of experiments, a mixture of chicken serum and embryo extract). A technique for testing micro-needles, to ensure that the polonium does not dissolve off into the medium, is also described.

Because the polonium source is not a "point", the dose rate when it is immersed in the medium cannot be calculated from, for example, measurements made in air with a counting ionization chamber; the measurements have to be recorded with both the micro-needle and the measuring apparatus immersed in the medium, or at least in a liquid in which alpha particles have a similar range. There are two possible techniques: to make the tracks of alpha particles visible in a photographic emulsion, or to count the tiny flashes of light emitted when particles strike a crystal of, for instance, zinc sulphide. The latter method has been used, because it permits working at a higher dose rate.

The customary unit for measuring doses of ionizing radiation is the rad. This is a measure of the mean amount of energy absorbed from the radiation by unit mass of the object which is being irradiated; one rad is a dose of 100 ergs per gram. When very small objects are irradiated with alpha particles, doses stated in rads can be misleading. Each single particle dissipates an appreciable amount of energy along its track. As a result, a dose which appears quite high when measured in rads may correspond to such a small number of particles, that parts of a cell which are amply large enough to be seen with a microscope may not be struck by any alpha particles at all. For example, a dose of 1000 rads of collimated alpha particles with energy 0.6 MeV corresponds to only one particle crossing each four square microns of the object. So, if a region of a cell which contained a granule with a cross-sectional area of one square micron were given a dose of this magnitude, it is very likely that the granule would not be traversed by any alpha particles at all. To emphasize the particulate nature of the radiation, in this series

of experiments dose rates are stated in terms of the alpha particle flux, expressed as particles per square micron per minute. Doses in rads are usually given as well, to allow comparison with other work.

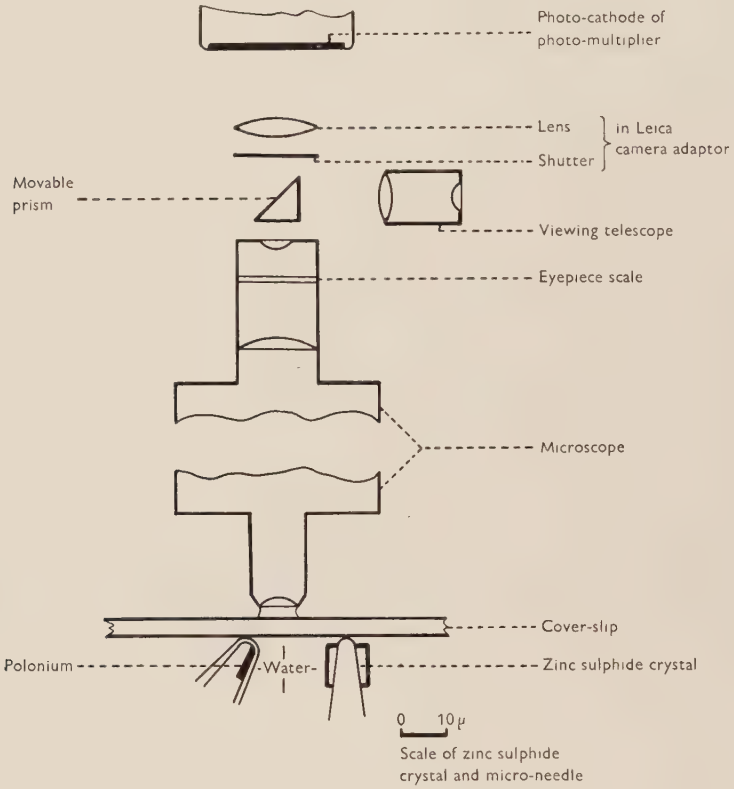

Fig. 1.—The optical side of the dosimetry apparatus (not to scale).

General technique.—A small crystal of silver activated zinc sulphide, approximately cubical and with sides about 10 microns long, is stuck to the tip of a micro-needle. This is placed at the point at which the dose is to be measured, with one face perpendicular to the direction in which the particles are travelling. The flash of light which is given off each time an alpha particle strikes the crystal is detected by a photo-multiplier (Fig. 1), and, if it is sufficiently large, is counted by a scaler. The apparent mean flux of alpha particles over the face of the crystal is then calculated. Since some of the particles do not yield enough light to be counted, the real flux is greater than this;

the method employed in calculating it is given in sections 1 and 2 of the Appendix. The values of linear energy transfer and range plotted in Fig. 9 enable the flux to be converted into the dose rate in rads per minute.

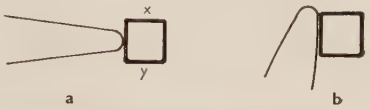

Fig. 2.—Zinc sulphide crystals mounted on a micro-needle. (*a*) Plan: as seen when measurements are being made; (*b*) side view.

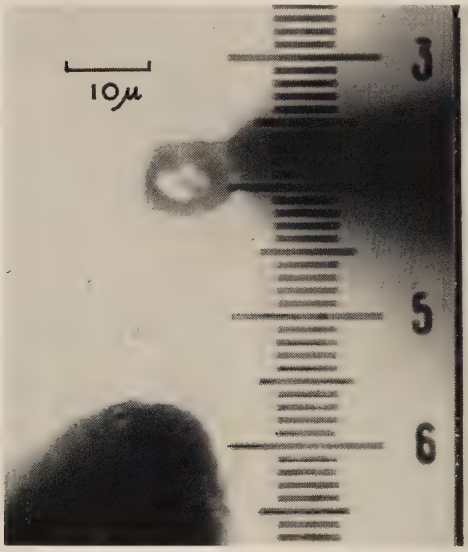

Fig. 3

Fig. 4

Fig. 3.—Zinc sulphide crystal, micro-needle and eyepiece scale. As seen when measurements are being made.

Fig. 4.—The same crystal as in Fig. 3; side view, showing the measured face which the alpha particles strike.

Mounting the counting crystals.—Tungsten micro-needles with tips of diameter 3–5 microns are prepared, and mounted in stainless steel tubes in the manner described previously [5]. The zinc sulphide crystals are stuck to the tips of the micro-needles with "Araldite D", a liquid adhesive which sets over a period of about 24 hours at room temperature when it is mixed with another liquid called Hardener 951.

The two liquids are mixed, and left for about an hour at room temperature (20°C). During this time, the mixture becomes slightly sticky. A very small drop of it is then placed near one edge of a $\frac{7}{8}$″ square coverslip. A thin layer of zinc sulphide crystals is spread over the remainder of the coverslip. It is inverted over a dry "chambre à huile" ([5], Fig. 4), and placed on the

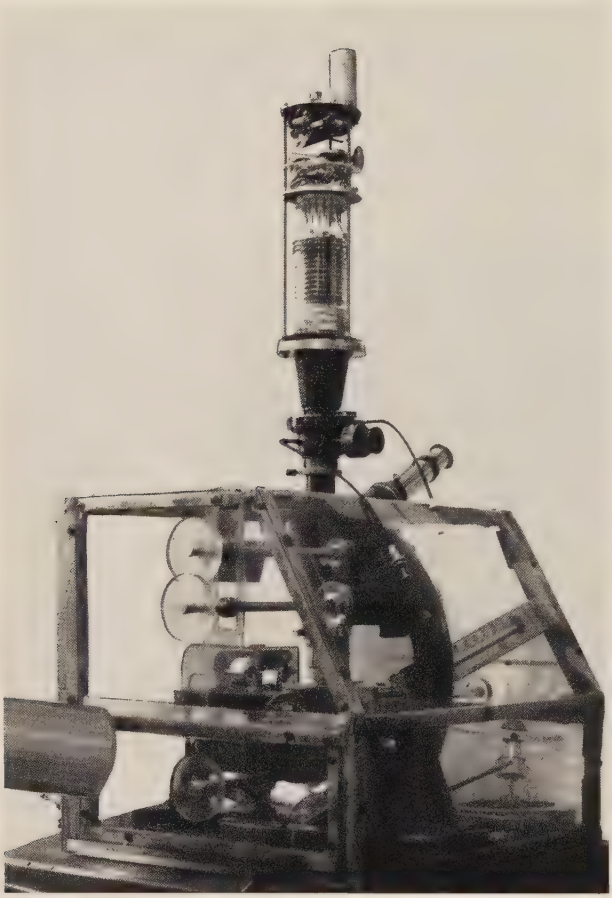

Fig. 5.—Photomultiplier (with casing removed) and viewing device mounted over microscope.

stage of a microscope. The micro-needle is introduced under the coverslip by means of the pneumatic de Fonbrune micromanipulator, and its tip is dipped into the Araldite. A thin film of the adhesive sticks to it. A suitable crystal, approximately cubical and with sides 7–11 μ in length, is selected and the needle is touched on to it, so that it adheres in the manner shown in Fig. 2*a*. It is left for 24 hours to allow the Araldite to set, heated at 100°C for 10 minutes to harden it further, and then examined. Crystals which are mounted in the manner shown in Fig. 2*b*, with the sides of the cube vertical and horizontal, are selected for trial. The areas of the two side faces (x and y in Fig. 2*a*) are estimated by measuring the lengths of the sides with a microscope fitted with an eyepiece scale. Figs. 3 and 4 are photographs of a typical crystal.

The viewing and counting system.—This is shown in Fig. 1. A bloomed Cooke achromatic ×95 oil immersion objective is used. The × 5 eyepiece contains a measuring graticule. Above this, a Leitz Leica camera adaptor contains a movable prism which enables the light to be directed either into a side viewing telescope, or upwards. Above the prism there is a camera shutter, and a camera lens which normally produces an image of the object

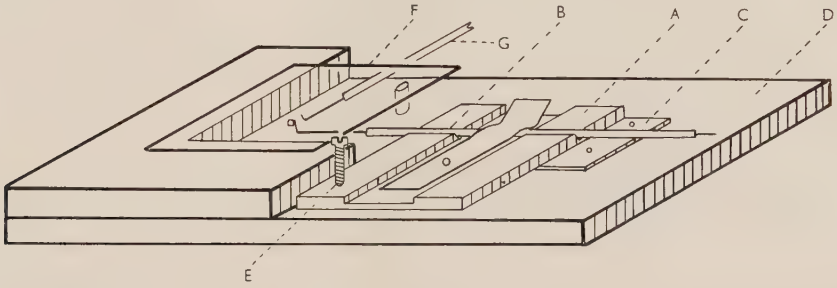

Fig. 6.—Apparatus for holding a mounted zinc sulphide crystal in contact with a coverslip. *A*, Clip holding micro-needle. *B*, which has the zinc sulphide crystal stuck to it. *C*, springy phosphor bronze strip fixing the clip *A* to the perspex base *D*. *E*, 8 B.A. screw, rotation of which bends *C* and raises or lowers the micro-needle *B*. *F*, Coverslip, supported 4 mm above the base by an L-shaped piece of brass and a small brass pillar. The space underneath it is filled with water. *G*, Micro-needle carrying polonium, which is being measured.

upon the film. The photo-cathode of an EMI 6255 photo-multiplier is placed approximately where the film would lie. Fig. 5 shows the photo-multiplier and Leica adaptor mounted on a microscope.

The pulses from the photo-multiplier are amplified and fed into the discriminator of a scaler. For measurements of alpha particle flux, this is set at 8 volts. The background, mainly due to thermionic emission from the photo-cathode, is then about one count per minute.

Experimental procedure.—The micro-needle which carries the zinc sulphide crystal is mounted in the clip *A* (Fig. 6). The coverslip *F* is placed in position, and the space under it is filled with distilled water. The apparatus is placed on the stage of a microscope fitted with a low power objective, and the crystal is raised until it touches the coverslip, by rotating the screw *E*. The micro-needle *G* which is to be measured is clamped in the holder for an auxiliary micro-needle on the de Fonbrune micromanipulator, and is placed in position under the coverslip and near the counting crystal, the technique described by de Fonbrune [3] being employed. The auxiliary micro-needle holder, which is adjusted by screw movements, is used here in preference to the pneumatic movement, because this tends to drift, probably because of small changes in temperature.

The bloomed × 95 objective is substituted for the low power objective, and the micro-needle *G* and the zinc sulphide crystal are brought into focus. The crystal is placed near the middle of the field, in a position such that its image is not obscured by the lines of the graticule in the eyepiece. *G* is placed at the required distance from it. The microscope lamp is switched off, the room is darkened, the prism above the eyepiece is swung out and the shutter opened (see Fig. 1), and the number of counts registered in (usually) one minute is determined. The shutter is closed, the prism swung back, and the distance between the needle *G* and the crystal is again measured. If it has changed by more than 0.5 μ, the count is repeated.

The Detection of Leakage of Polonium from Micro-needles

Sometimes, the chromium coating on a micro-needle is slightly porous. When the tip of such a needle is immersed in the tissue culture medium, some of the polonium may escape. With the more powerful needles, giving off perhaps a million alpha particles per minute, if even a fraction of one per cent of the polonium entered the tissue culture medium and stayed near a cell that was being irradiated, the cell might be subjected to continuing irradiation after the needle had been removed, and parts of it which should not have been irradiated might receive significant doses. Loss of a small fraction of the polonium cannot conveniently be detected by measuring the amount of polonium on a needle before and after an experiment; it is necessary to search in the culture medium for polonium which may have escaped into it.

It is not technically feasible to perform a direct test, and look for polonium in the medium each time a micro-needle has been used to irradiate a cell. Instead, needles are tested immediately after they have been made, and at intervals during their life.

Testing technique.—A zinc sulphide screen with a sharply defined edge is made on a $\frac{7}{8}''$ square coverslip (Figs. 7*a* and 8). A drop of tissue culture me-

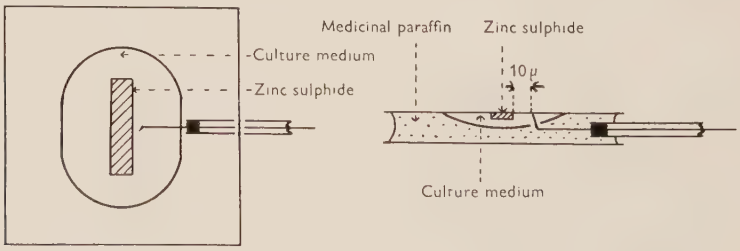

Fig. 7.—Test for leakage of polonium. (*a*) Plan; (*b*) side view.

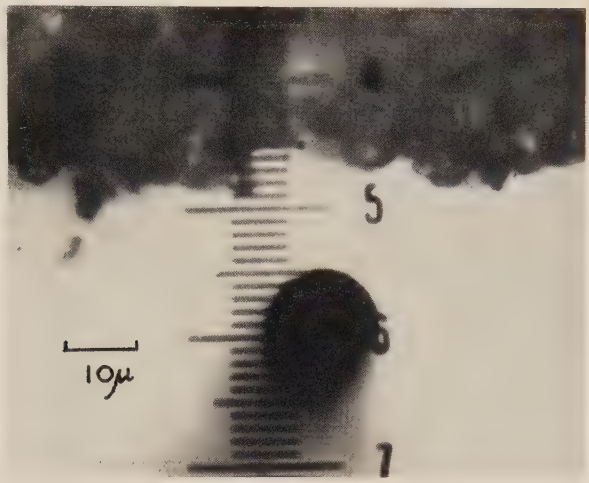

Fig. 8.—Micro-needle and zinc sulphide screen, as seen when tests are being made for leakage.

dium is placed on the coverslip; this is inverted over medicinal paraffin contained in a "chambre à huile", on the stage of a microscope enclosed in a warm box at 37°C. The micro-needle which is to be tested for leakage is introduced under the coverslip, placed at a distance of 10 microns from the edge of the zinc sulphide, and left there for 5 minutes (Fig. 7 *b*). During part of this time, the light from the scintillations is allowed to strike the photomultiplier, to check that the micro-needle is opposite a sensitive part of the zinc sulphide layer. This check was particularly important, since the sample of zinc sulphide which was used did not have uniform sensitivity.

Then the needle is removed, and the zinc sulphide which has been near the micro-needle is "watched" with the microscope and photomultiplier for five minutes, to see whether any scintillations caused by alpha particles from polonium which has been left behind can be detected.

The optical arrangements are the same as those described on p. 538 but the discriminator level is increased to 16 volts, which reduces the background to an average of one count in 15 minutes. If more than one count is registered during the five-minute "watching" period, or if the size of a single pulse is such as to suggest that it originated from an alpha particle, the needle is discarded or, if there is uncertainty, the test is repeated. The results obtained suggest that, if polonium is leaking from a needle at all, enough usually comes off to register a number of counts during the five minute watching period. Experience has shown that the needles will stand any reasonable amount of downward pressure from coverslips, which produces a compressional stress. If, on the other hand, a needle catches on a coverslip

as it is being inserted in a "chambre à huile", the chromium coating, which is weak in tension, invariably becomes porous.

Making the zinc sulphide screens.—A pinch of zinc sulphide is placed on the middle of a $\frac{7}{8}$" square coverslip. A drop of a mixture of equal parts of D.P.X. mountant (B.D.H.) and xylene is placed on top of it, and left to dry for at least 24 hours. Just before it is to be used, the edges are trimmed with a razor blade, and the surplus D.P.X. and zinc sulphide are peeled off, leaving a strip 0.5–1 cm long by 1–2 mm wide.

SUMMARY

A method is described for measuring the dose rate of alpha radiation near a polonium-tipped micro-needle, which can be used to irradiate parts of a single cell. A zinc sulphide crystal, stuck to another micro-needle, is placed near it; the scintillations produced when alpha particles strike this are detected by a photo-multiplier mounted over a microscope. A technique for detecting leakage of polonium from micro-needles is described. The method of calculating the particle flux from the measurements is set out in an Appendix.

Messrs. Oliver and Boyd have kindly given permission for Fig. 3, from *Advances in Radiobiology* (1957), to be reproduced.

I should like to thank Dr. C. W. Gilbert and Dr. J. P. Keene for assistance with apparatus and advice regarding the electronic side of the work. I am indebted to Mr. J. Lister and his staff for their patient construction of most of the apparatus. I am grateful to the Royal College of Surgeons for a Prophit Studentship, the British Empire Cancer Campaign for a Junior Fellowship, and to the Royal College of Physicians for a Saltwell scholarship. And I have to thank Dr. Edith Paterson for her encouragement.

APPENDIX

The alpha particle energy required to register a count

The photomultiplier produces some pulses, even when its cathode is not illuminated. These have origin mainly in thermionic emission from the photo-cathode, and most of them are smaller than the pulses produced by flashes of light from the zinc sulphide. They are cut out by the discriminator, which precedes the scaler, and will only pass pulses which are larger than a certain value. If the flash of light produced when an alpha particle strikes the zinc sulphide crystal is to be registered as a count, it must then produce a pulse which is large enough to trigger the discriminator. To achieve this, the particle must expend more than a certain minimum amount of energy in the crystal. For given electronic conditions, the energy required varies somewhat from crystal to crystal, partly, at least, because some crystals have a

layer of Araldite D on their upper surfaces, which absorbs some of the light emitted. Accordingly, it has to be measured for each crystal.

Measurement of this energy is complicated by two factors:—

(1) The variation of the linear energy transfer ($-dE/dx$) of a particle with its energy (Fig. 9).
(2) The fact that alpha particles of given energy have a smaller range in zinc sulphide than in water.

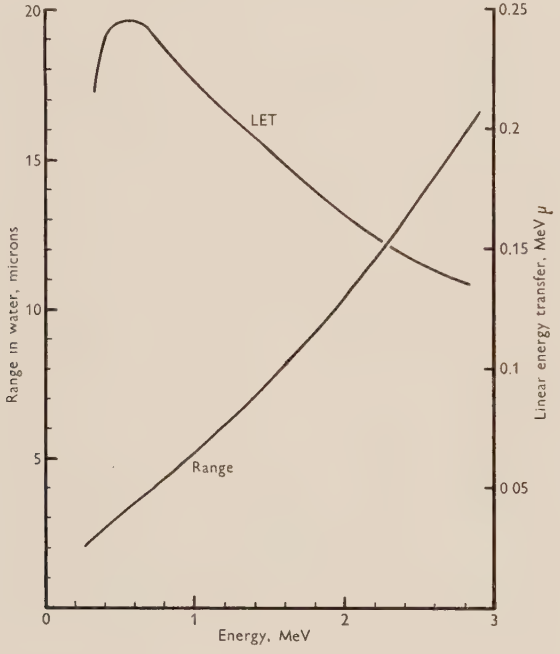

Fig. 9.—Range–energy and linear energy transfer (LET)–energy curves for alpha particles in water.

The method used is described in the paragraphs which follow. We shall assume that:—

(1) The amount of light emitted by a zinc sulphide crystal when an alpha particle strikes it is directly proportional to the energy dissipated in the crystal. Broser and Kallmann [2] found this to be so, at least for particles with energies between 0.25 MeV and 2 MeV.
(2) The size of the pulse which enters the discriminator is directly proportional to the amount of light emitted by the crystal. Statistical fluctuations in the gain of the photomultiplier will be ignored.
(3) The range–energy relation for alpha particles in water is as given in Fig. 9, which was calculated from the data of Jesse and Sadauskis for alpha particles in air [4], and the stopping power of water (1.47 at 25°C) given by Aniansson [1]. Possible variation of the stopping power with energy has been neglected.

Referring to Fig. 10, we shall assume that the crystal is cubical. To simplify the calculations, we shall also assume that the polonium is concentrated at O, and when we are considering the length of the path of a particle in the crystal, we shall imagine that its direction is parallel to OP, the normal from O to the face A, even if it is following a path to one of the corners.

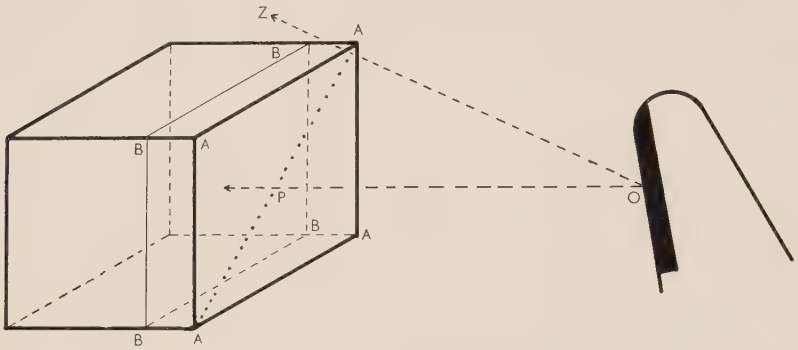

Fig. 10.—Micro-needle and counting crystal (see text).

Suppose that, when the discriminator is set at V volts, an energy E_v has to be dissipated in the crystal to register a count. E_v can be measured in the following manner:—

As the distance OP from the micro-needle to the crystal is increased, the counting rate will drop. Suppose it reaches zero when $OP = l$. At this point, the particles with the longest range are dissipating an amount of energy in the crystal which is just too small to trigger the discriminator; that is, just less than E_v (Fig. 11*a*). From here, let the crystal be moved a distance d towards the needle (Fig. 11*b*). Suppose the maximum height of the pulses now reaching the crystal is V_m volts. These pulses will come from the particles with longest range, mentioned above; before the crystal was moved, they dissipated energy just E_v in it. If E_d is the energy lost by one of these particles in passing through water from a distance $(l - d)$ to a distance l, then the pulses of height V_m result from an energy $(E_v + E_d)$ dissipated in the crystal.

From the assumed proportionality between energy dissipation and pulse size, we now have

$$V/V_m = E_v/(E_v + E_d). \tag{1}$$

We calculate E_v by successive approximation. To begin with, we assume that E_d is equal to the energy dissipated in a distance d at the end of the range of the particle, instead of in a distance d ending perhaps 2 3 μ from the end of the range. These energies are different because of the variation of the linear energy transfer with particle energy (Fig. 9). The measured value of d is used in reading off a first value of E_d from the end of the range–energy relation (Fig. 9), and an approximate value of E_v is calculated by substituting this in eq. (1) above.

Now let r_e be the range in the crystal corresponding to an energy E_v at the end of

the track of a particle, and s be the ratio of the range of a particle in water to the range of a particle with the same energy in zinc sulphide. From the approximate value of E_v, an approximate value of sr_e can be read off from Fig. 9. Knowledge of sr_e enables E_d to be calculated at the correct part of the track of the particles, again using Fig. 9. A more accurate value of E_v can then be calculated from eq. (1). This

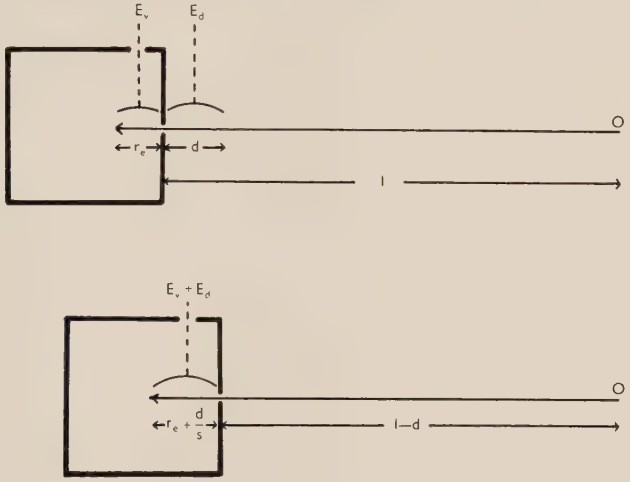

Fig. 11.—Energy dissipation in a zinc sulphide crystal (see text).

second approximation is usually as accurate as the measurements from which it is derived.

To calculate s, the range of polonium alpha particles in zinc sulphide was estimated from stopping powers given in [6], by means of the Bragg law. The range came to 21 μ, which gives a value 1.9 for s.

Correction of the dosimetry measurements

When the crystal is near the end of the range of the particles, it registers counts from those particles which have a range of at least r_e in it; i.e. those which have an energy of at least E_v when they strike it. A particle with a residual range r_e in the crystal will have a residual range sr_e in water. Accordingly, near the end of the range, measurements of the alpha particle flux refer to a distance $(l+sr_e)$ from the needle. They have to be further corrected for the small decrease of flux with distance, between $(l+r_e)$ and $(l+sr_e)$. We assume that this follows the inverse square law, and so multiply the measured fluxes by $(l+r_e)^2/(l+sr_e)^2$. This correction has been applied to all measurements made at points less than 10 μ from the apparent end of the range.

When the crystal is closer to the micro-needle, the correction which has to be applied is simpler. It still counts those particles which dissipate an energy of at least E_v in it. Suppose they have to traverse a distance r_v in the crystal to achieve this, and, referring to Fig. 10, let B be a plane at a distance r_v from the face A. The crystal

will not count all the particles which cross A; some of those which pass near its edge (e.g. along OZ) will leave it before they have covered the distance r_v. On the other hand, it will count all the particles which cross B, and provided that the crystal is sufficiently close to the micro-needle for no particles to be stopped between A and B, the flux at the latter plane will not be changed if the crystal is removed and replaced by water. Accordingly, measurements made when the distance between the needle and the crystal is l, actually give the flux at a distance $(l + r_v)$ from the needle.

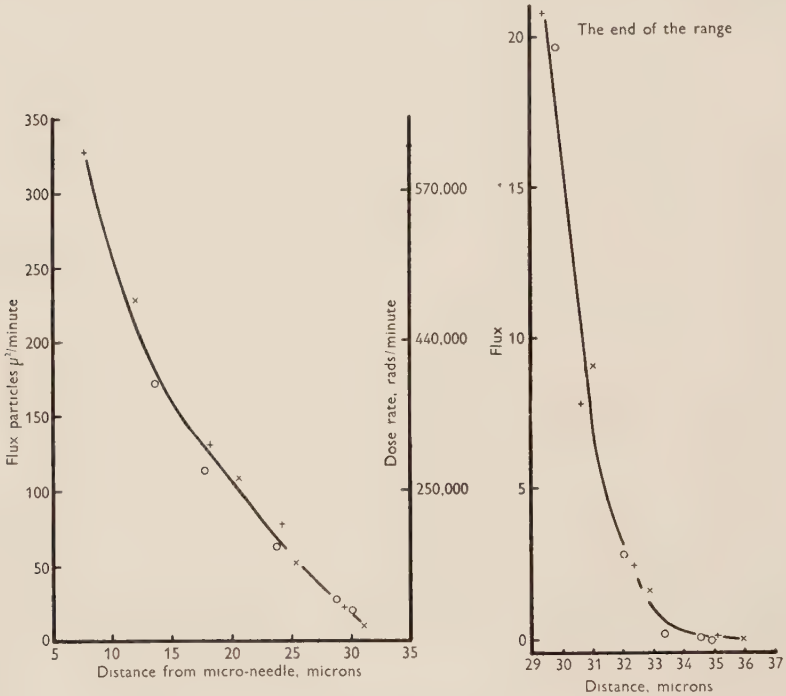

Fig. 12.—Corrected particle flux–distance curves.

Points	Crystal	E_v	sr_e
0	1	0.26	2.0
+	2	0.42	2.7
×	2[a]	0.76	4

[a] Measurements made two months after those in the line above, with slightly different electronic conditions.

When the maximum range of the particles from a given micro-needle has been measured, the value of r_v at various distances from it can be calculated from E_v and the range–energy relation (Fig. 9).

The validity of these methods for calculating the dose has been tested by measuring the same needle three times, varying r_e by using two different crystals and by altering the electronic conditions. The corrected fluxes from one set of experiments are shown

in Fig. 12, and it will be seen that the three sets of measurements are in satisfactory agreement.

Crystals for which the value of sr_e is not greater than 4 μ have been selected for use in this work, since some of the assumptions made in calculating r_e fail to hold when the correction required is large.

To give some idea of the orders of magnitude we are dealing with, the left-hand graph of Fig. 12 gives dose rates in rads per minute as well as particle fluxes.

Fig. 13.—(*a*) Well-shaped micro-needle, showing a sharply defined edge; (*b*) bent micro-needle, with no edge from which to measure.

The importance of the shape of the micro-needle which carries the polonium

If accurate, or indeed any dosimetry is to be possible, it is essential that the micro-needle which carries the polonium should have a sharp, well defined edge, from which distances can be measured. During the dosimetry measurements, and all the irradiations carried out so far, the micro-needle has been observed with oil immersion objectives, which have a depth of focus of a fraction of a micron. If a micro-needle is to be usable, it must have a size and shape similar to that shown in Fig. 13*a*. Here, if the objective is focussed on the plane *A*, a cross-section of the tip of the micro-needle will be seen, with a ring surrounding it which corresponds to the out of focus part of the needle at *P*. If the microscope is focussed down, the edge will become sharp when the plane *B* is in focus. Distances can then be measured accurately from *P*. It is important that *P* should not be too far below the level of the coverslip; when the micro-needle is used to irradiate a cell, the part of the cell which is being studied must be in focus at the same time as *P*. The same applies to the edge of the counting crystal, when a micro-needle is being measured. A cell at metaphase and a counting crystal are drawn to scale, in Fig. 13*a*, to compare with the micro-needle.

Needles shaped like the one shown in Fig. 13*b* are almost useless. This needle does not show a sharp edge; as the microscope is focussed down, its apparent edge simply moves continually towards the cell. The very tip of the needle (*T*) is the only possible point from which to measure doses, and this is not well defined. An additional trouble is that a needle of this kind will disturb the illumination of the cell.

Needles cannot easily be bent after they have been plated. The chromium coat makes them brittle, and they usually break if one attempts to bend them.

Other measuring apparatus

Besides the scintillation counting equipment, two counting ionization chambers have been used, in combination with a linear amplifier and a discriminator and scaler. Both are parallel plate chambers, with wire gauze outer electrodes, through which the particles enter. One is a simple chamber, and is used to ascertain approximately how much polonium there is on a given needle. It tends to become contaminated,

and accordingly is made so that the gauze outer electrode can be replaced easily. The other chamber has a circular collecting electrode, 2.5 cm in diameter, with a guard ring. Its high potential electrode is made of 100 mesh phosphor bronze gauze, and the chamber is 2 mm deep. The micro-needle which is being measured is held on a slide with a screw adjustment, calibrated in tenths of a millimetre. This chamber is used to supplement the test described above in section *B*, to provide a further check on the possibility of leakage of polonium from micro-needles. Each needle is measured with the chamber when it has been made, and at intervals afterwards. Any gross loss of polonium (e.g. 10 per cent) would be detected. So far, with measurements taken over periods up to 200 days, no loss has been detected in any micro-needles which have passed the more sensitive test mentioned above.

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REGULATION OF RESPIRATION IN HEART SARCOSOMES

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Recently, intact heart muscle mitochondria (sarcosomes) have been isolated from several animal species in different laboratories. Investigations of these respiratory granules have demonstrated the presence of many oxidative enzymes and of the oxidative phosphorylation process [2, 5, 9, 15, 16]. Although these particles have been shown to carry out oxidative phosphorylation, they have been termed "loosely-coupled" because of the presence of enzymes which break down newly formed phosphate esters. Such preparations differ from "tightly-coupled" particles in which it is unnecessary to add phosphate-trapping enzymes to demonstrate net adenosine triphosphate (ATP) synthesis.

As an approach to a study of the mechanism of action of the respiratory pigments of intact phosphorylating sarcosomes, an investigation was made of some of the factors which interfere with the direct measurement of oxidative phosphorylation.

In 1952, Lardy and Wellman [14] observed that a large increase in the rate of respiration occurred on addition of either phosphate acceptor, adenosine diphosphate (ADP), or uncoupler, 2,4-dinitrophenol (DNP) to "tightly-coupled" mitochondrial preparations incubated in suitable suspending media and substrates. The ratio of the slow respiration obtained in the presence of substrate alone to the accelerated rate of respiration caused by ADP addition has been used by Chance and Williams [3, 4] to measure oxidative phosphorylation by means of a rapid and simple polarographic assay. This method could not be utilized with loosely-coupled mitochondrial systems because no cessation of the rapid respiration initiated by ADP occurred, because ATPase action regenerated ADP.

It has now been found possible to apply this polarographic method for assay of oxidative phosphorylation in heart sarcosomes of several animal species. This report also describes some general characteristics of the kinetics of oxygen consumption of sarcosomes.

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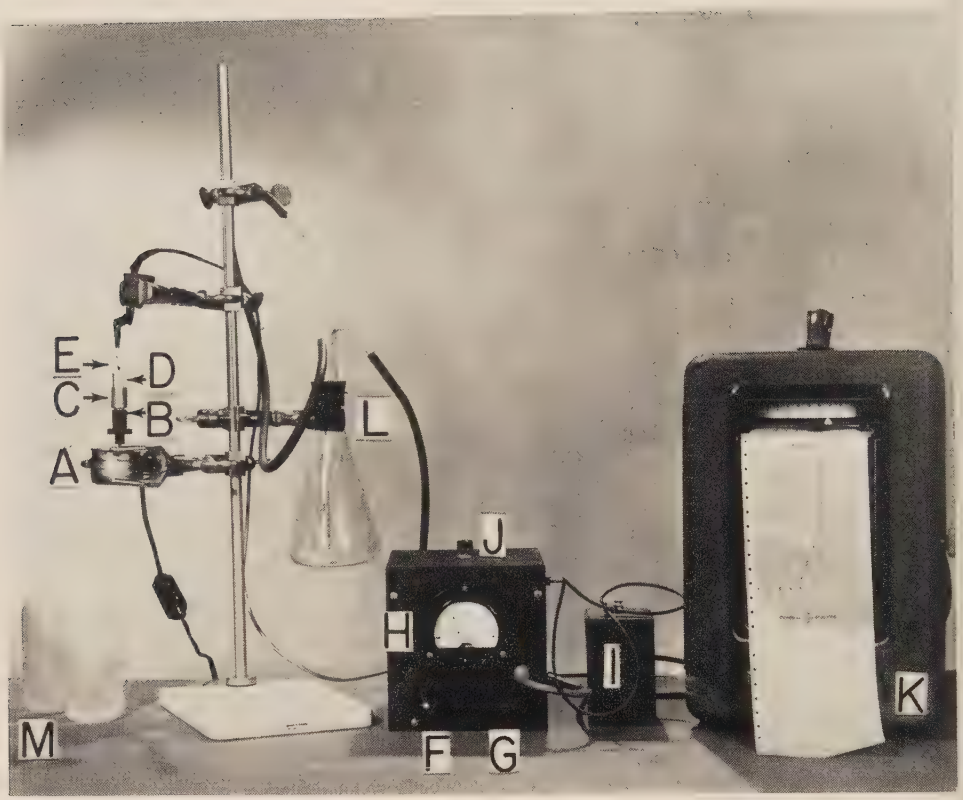

Fig. 1.—The O_2 Electrode Apparatus. *A*, synchronous motor for turning cuvette; *B*, plastic bushing to prevent heat transfer from the motor to the cuvette; *C*, cuvette holder; *D*, cuvette with 2.0 ml capacity; *E*, electrodes; *F*, polarizing voltage control; *G*, sensitivity control; *H* voltmeter to read polarizing voltage applied; *I*, battery (1.5 Volt) for polarizing voltage; *J*, pen adjustment for recorder; *K*, general Electric CE-5 amplifier potentiometric recorder; *L*, vacuum apparatus for rinsing out contents of cuvette at the end of experiments; *M*, plastic wash bottles.

METHODS

Preparations.—Heart muscle sarcosomes were prepared from animals by the procedure of Cleland and Slater [5], using a sucrose (0.32 *M*)-versene (0.001 *M*) suspending medium at pH 7.5.

Analyses.—Inorganic phosphate was determined by the butanol extraction method described by Ennor and Stocken [7]. ATPase was assayed by the method of Zetterström and Ernster [24]. Sarcosome protein was calculated by multiplying Kjeldahl nitrogen analyses by 6.25. The ADP used was the chromatographically pure preparation supplied by the Sigma Chemical Company; the concentration was determined by using the extinction coefficient of 16 $cm^{-1} \times mM^{-1}$ (at 260 $m\mu$) [4].

Measurement of O_2 utilization.—Oxygen consumption was measured polarographi-

cally with an oxygen electrode (Pt-Ag couple) of the "open" type described by Davies and Brink [6]. The apparatus is illustrated in Fig. 1. The electrode was immersed in a medium containing traces of chloride ions, and a polarizing voltage of 0.60 to 0.75 V was applied. The current at the platinum cathode, which is proportional to the concentration of dissolved oxygen in the medium, was measured polarographically by a General Electric (Model CE-5) amplifier-recorder. To insure rapid equilibration of changes in oxygen concentration, the cuvette in which the reactions occurred was placed in a holder turned by a small synchronous motor. The shaft of the motor was equipped with a plastic bushing which prevented heat transfer; the temperature of the reaction mixture (25° to 26°C) remained constant in the several minutes required to complete an experiment.

The sensitivity of the electrode did not drift significantly when a series of experiments were made with the same suspending medium. It was found convenient to immerse the electrodes to the midpoint of the reaction mixture. After completion of an experiment, the contents of the cuvette were removed by suction and the cuvette was washed several times with distilled water and finally with sucrose. Thus, it was unnecessary to change the position of the electrodes, providing for constancy of conditions.

A linear relationship existed between oxygen consumption and sarcosome concentration when the protein concentration varied between 0.5 and 10.0 mg/ml. Rates of O_2 consumption are reported in μ moles consumed per second per liter (μM/sec).

RESULTS

Polarographic assay of oxidative phosphorylation.—A control experiment with rabbit heart sarcosomes, shown in Fig. 2, illustrates the method for assay of respiration and oxidative phosphorylation. The experiment was

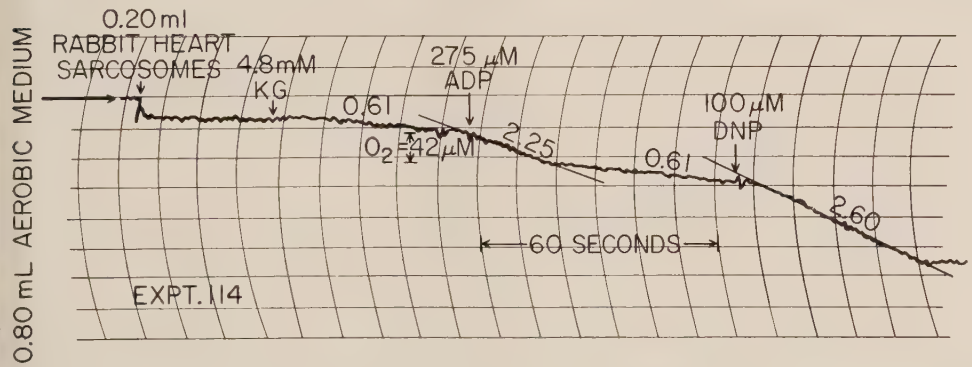

Fig. 2.—Polarographic assay of oxidative phosphorylation during the respiration of KG in rabbit heart sarcosomes. The air-saturated aerobic medium contained: 0.32 *M* sucrose, 0.01 *M* KCl, 0.02 *M* phosphate buffer, and 0.001 *M* disodium ethylenediamine tetraacetic acid, at pH 7.5. The concentration of the other additions are shown except for the sarcosome suspension which contained 2.5 mg protein. Time moves from left to right and a downward deflection of the curve indicates O_2 consumption. The numbers above the curve refer to the calculated rate of O_2 consumption in μM per second. Temperature, 26°C.

begun by the addition of the aerobic suspending medium to the rotating cuvette. The initial O_2 concentration is indicated by the level of the recorded trace. An aliquot of the sarcosome suspension was then added, and the next portion of the trace shows that endogenous O_2 consumption was very small since no significant deflection of the trace is observed. After 30 seconds, α-ketoglutarate (KG) was added to the reaction mixture, causing, after a 15 second lag period, an increased rate of O_2 consumption as indicated by the downward deflection of the trace. A lag phase in the respiration of this substrate has also been reported for rat heart sarcosomes [2, 16]. The rate of respiration with KG was calculated to be 0.61 μM O_2 utilized per second.

The addition of a phosphate acceptor, adenosine diphosphate (ADP), is seen to cause a 3.7 fold increased rate of respiration, which proceeds linearly for 25 seconds and then decreases by a factor of 3.7. When the original rate of KG respiration is restored, it is presumed that the added ADP has been converted to adenosine triphosphate (ATP). Thus it can be calculated that 42 μM O_2 (or 84 μ atoms per liter) were consumed during the conversion of 275 μM ADP to ATP, giving an ADP/O ratio of 275/84 or 3.2.

Since the ADP/O ratio is equivalent to the P/O rates in tightly-coupled oxidative phosphorylation systems [3], the finding of an ADP/O ratio of 3.2 for KG is in agreement with that found generally with this substrate [15].

Many cycles of initiation and cessation of rapid respiration by addition of ADP can be repeated in a single experiment yielding multiple determinations of the rate and efficiency of oxidative phosphorylation with this substrate using this assay system in heart sarcosome preparations. Uncoupling of respiration also causes an increased rate of respiration, but here no cessation of the rapid respiration occurs. Thus in the present experiment the addition of 100 μM 2,4-dinitrophenol (DNP) uncouples phosphorylation, and increases respiration of KG by a factor of 4.2-fold. When the O_2 in the reaction mixture is exhausted, the trace is horizontal.

Sarcosome preparations isolated from rabbits, rats, mice, guinea pigs, monkeys, pigs, dogs, frogs and toads with the sucrose-versene medium have thus far been shown to exhibit tightly-coupled oxidative phosphorylation during the respiration of KG with ADP/O ratios greater than 3. The effects of ADP, DNP, and various substrates in these different sarcosome preparations were likewise similar. Some typical data on the nature of the respiration obtained with different substrates and ADP is given in Table I for a rat preparation. Although succinate was characteristically the substrate oxidized at the most rapid rate by heart muscle preparations, respiratory stimulation with this substrate was the poorest. Other substrates, such as KG and glutamate,

which are respired more slowly in the absence of ADP, were stimulated very much more when ADP was added (see discussion). In other experiments, the DNP stimulation of the substrate respiration paralleled qualitatively the respiratory stimulation obtained with ADP [16]. Half-maximal stimulation of respiration was obtained with approximately 40 μM ADP or DNP.

TABLE I. *Effect of ADP on respiration of rat heart sarcosomes.*

The reaction mixture (1 ml) contained sucrose (0.32 M), versene (0.80 mM), P (0.016 M at pH 7.5), KCl (0.80 mM), and sarcosome suspension (1.23 mg protein). The ADP concentration was 360 μM, added as indicated.

Substrate	Concentration (mM)	O_2 consumed (μM/sec.) −ADP	+ADP	Respiratory stimulation
Succinate	10	1.51	3.63	2.4
β-Hydroxybutyrate	2	0.14	0.69	4.9
α-Ketoglutarate	5	0.21	2.74	13.1
Glutamate	20	0.12	1.51	12.6
Pyruvate	2	0.07	0.27	3.9
Acetate	2	0.07	0.41	5.9

ATPase.—Since it was customary to add magnesium ions to phosphorylating media [5, 22, 23], our initial experiments with sarcosomes isolated in 0.32 M sucrose were carried out in presence of magnesium [16]. Under these conditions it was found that ADP caused an acceleration of substrate respiration, but only a partial cessation of this rapid phase of respiration occurred, yielding low ADP/O ratios. This finding suggested that ATPase's in the preparation may have been regenerating ADP from ATP, thus preventing the complete cessation of the rapid phase of respiration. Indeed a magnesium and calcium activated ATPase was found present in sarcosome suspension. The ATPase activity was found to be 6, 58, and 55 μ moles ATP hydrolyzed/liter/minute/mg sarcosomal protein for control, $MgCl_2$ (5 mM) and $CaCl_2$ (5 mM) respectively when assayed according to the procedure of Zetterström and Ernster [24]. The activity was rather stable on storage at 2°C, only 24 per cent being lost after four days. The small amount of endogenous ATPase was virtually eliminated if the sarcosomes were prepared in sucrose media containing 0.001 M Versene. Under these conditions the magnesium content of sarcosomes was found to be 0.039 and 0.013 μ moles/mg protein for sucrose and sucrose-versene respectively [17].

ATPase vs. oxidative phosphorylation.—Although it has been possible to obtain high ADP/O ratios with KG and other substrates by eliminating Mg ions and thus lowering the ATPase activity in sarcosome suspensions, it was found that even under these conditions low values are obtained during succinate respiration. A typical experiment is shown in Fig. 3 in which the slow

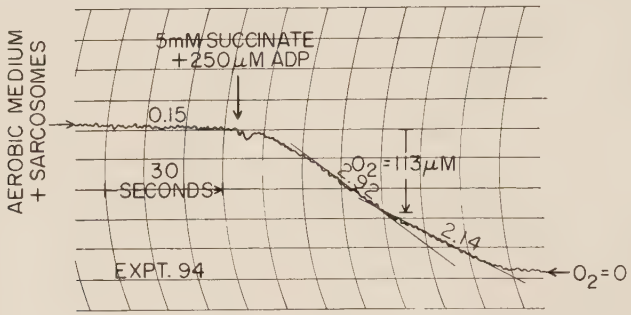

Fig. 3.—Effect of ADP on succinate respiration. The same conditions as described in Fig. 2 were used in this experiment. Time moves from left to right, with the horizontal scale calibrated for 10-second intervals.

rate of endogenous substrate oxidation (0.15 μM O_2/second) is recorded. The addition of succinate and ADP simultaneously results in a 20-fold increase in respiration, which decreases after approximately 40 seconds. The 113 μM O_2 uptake in this 40-second interval corresponds to an ADP/O ratio of 1.1 for succinate. With the same sarcosome suspension, the O_2 consumption with succinate in the absence of ADP was 1.20 μM/second. Thus, even in the absence of Mg ions there is no sharp cessation of the rapid respiration caused by ADP, during the respiration of succinate.

It seemed possible that succinate itself may cause an increased hydrolysis of ATP in sarcosome suspensions. This hypothesis could easily be tested with succinate because respiration of this substrate can be inhibited by malonate. The formation of P from ATP in sarcosome suspensions was followed as a function of succinate concentration in the presence and absence of malonate (Fig. 4). With the concentration of malonate employed, no measurable uptake of oxygen occurred with the range of succinate concentrations used. Also, malonate itself does not influence the reaction. The data show clearly that the rate of ATP hydrolysis is a function of succinate concentration when malonate is present. In the absence of malonate, increasing succinate concentrations decrease the P content of the reaction mixture. This is due to the balance between P formed by ATP hydrolysis and P uptake during oxidative phos-

phorylation (the latter being more active). Thus, the decreasing P formation in the presence of increasing succinate concentrations (or the equilibrium of the process) would appear to be related to the higher respiration shown in Fig. 4 with increasing succinate concentrations. On the other hand, the ATP hydrolysis caused by succinate occurs in the absence of respiration.

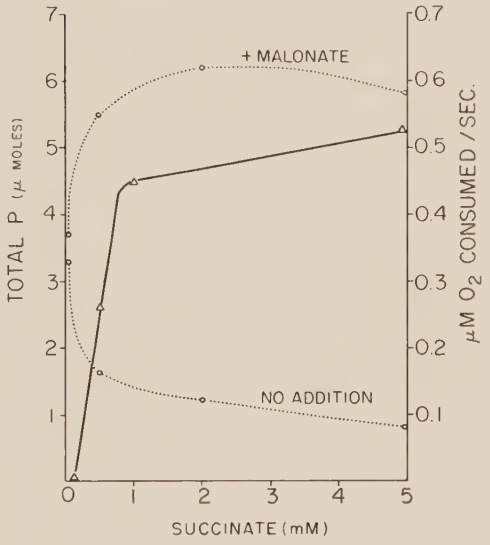

Fig. 4.—Effect of succinate concentration on ATP hydrolysis in the presence and absence of respiration. The reaction mixture (2 ml) contained tris(hydroxymethyl) aminomethane (0.05 *M* at pH 7.6), KCl (0.01 *M*), ATP (7.5 m*M*), sucrose (0.208 *M*), versene (0.65 m*M*), and malonate (0.05 *M*) where indicated in the figure. The sarcosome suspension contained 1.88 mg protein. Aliquots of the reaction mixture were tested for *P* after 60 minutes.

DISCUSSION

Respiration and coupled phosphorylation were studied in heart sarcosomes of nine animal species by means of the direct polarographic measurement of dissolved O_2 in reaction mixtures. An equilibrium between the rate of oxidative phosphorylation and ATPase activity was observed, i.e.

$$\text{ADP} + \text{P} \rightleftharpoons \text{ATP}.$$

This equilibrium could be shifted in the direction of oxidative phosphorylation by omitting Mg ions to limit ATPase. This sarcosome ATPase appears to be different from the Mg-activated enzyme of muscle [12] in that the latter enzyme is inhibited by Ca ions and is unstable on storage, whereas the sarcosome enzyme is activated by both cations and is relatively stable on storage. By studying respiration in Mg-free media it was readily shown that high ADP/O ratios were obtained upon addition of ADP, except when succinate was used as a substrate. These experiments were carried out with sarcosomes

prepared in sucrose media containing a small amount of versene. By preparing sarcosomes in sucrose media containing versene the Mg content of sarcosomes was lowered about 3-fold over that present in preparations made in sucrose only. The bound Mg-content (0.013 μ moles per mg protein) is apparently sufficient for tightly-coupled oxidative phosphorylation, at least for the short time required for experiments with the polarographic method.

It has been reported [8] that difficulties are encountered in the measurement of oxidative phosphorylation during succinate respiration. Similar results were obtained in these experiments, even in Mg-free media. After the addition of ADP to sarcosomes respiring succinate a 2–4 fold increased respiration occurred; this rapid phase of respiration only partially decreased even though an amount of ADP was added which should have been completely phosphorylated. It was shown that the ADP was not being completely removed through the action of an enzyme other than the MG-activated ATPase, which was hydrolyzing ATP (Fig. 4). Since this rate of hydrolysis of ATP was dependent on the concentration of succinate, it may be that the difficulties encountered in the polarographic assay of oxidative phosphorylation during succinate respiration are due to the action of the so-called P-enzyme [10] or thiokinase [18] which catalyze the over-all reaction

$$\text{Succinate} + \text{ATP} + \text{Coenzyme A} \rightleftharpoons \text{Succinyl} \cdot \text{Coenzyme A} + \text{P} + \text{ADP}.$$

Another point worthy of consideration is the poor stimulation of succinate respiration by ADP when compared to the higher stimulations obtained with other substrates (Table I). It may be that this is also the result of the succinate-linked hydrolysis of ATP which would be envisaged to act by preventing the removal of phosphate acceptors, thereby causing a more rapid rate of respiration on succinate. The intramitochondrial regulation of respiratory rate by ADP has been reported on by Siekewitz and Potter [19] who showed that when ATP was added to mitochondria there is a slow breakdown of the ATP to provide phosphate acceptors which caused a low level of respiration.

The results of Siekewitz and Potter are in agreement with the low endogenous ATPase activity and endogenous respiration (Fig. 3) of the heart sarcosomes.

SUMMARY

The respiration of suspensions of heart sarcosomes was investigated using a polarographic technique for measuring rapidly changes in oxygen concentrations. Conditions were defined for studying the kinetics of oxygen utilization during substrate oxidation, oxidative phosphorylation, and when oxida-

tion was uncoupled from phosphorylation. The role of adenosine triphosphatase activity in the regulation of these processes was reported and discussed.

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ÉTUDES SUR LE DÉTERMINISME DE LA MORPHOGÉNÈSE D'UN TRYPANOSOME

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Dans un travail récent, Hutner [8] a souligné les nombreux avantages de l'utilisation des protozoaires dans l'étude de la morphogénèse des métazoaires. Les trypanosomides méritent à cet égard un intérêt particulier. En effet, ils constituent une série phylogénétique, allant des strigomonas aux trypanosomes parasites des mammifères, dans laquelle forme et métabolisme évoluent parallèlement. On a montré par exemple [3] qu'au cours de la phylogénèse de cet ordre, un mécanisme respiratoire sensible au cyanure est progressivement remplacé par un mécanisme insensible et que cette évolution biochimique s'inscrit assez exactement dans l'ordre taxonomique des trypanosomides telle qu'il fut établi par Hoare. Un autre intérêt de ce matériel réside dans son cycle biologique: On sait que ce cycle fait passer les trypanosomes par deux formes principales : (1) la forme monadienne ou crithidia, (2) la forme trypanosome (sanguicole). La première se développe dans les organes digestifs de l'hôte invertébré. La seconde est propre à la circulation de l'hôte vertébré et se caractérise essentiellement par la présence d'une membrane ondulante et par la position postérieure du kinétoplaste. La forme crithidia d'un trypanosome est morphologiquement semblable aux trypanosomides monomorphes primitifs des genres *leptomonas* et *crithidia*. Il est probable que cette similitude existe également sur le plan biochimique et que, sous leur forme crithidia, les trypanosomes ont un métabolisme moins spécialisé par le parasitisme que sous leur forme sanguicole.

La connaissance précise du déterminisme de la transformation des crithidias en trypanosomes pourrait être précieuse dans l'étude des processus de différentiation et de dédifférentiation cellulaire. Ces transformations peuvent être induites par le milieu extérieur et étudiées *in vitro*. En effet nous avons montré récemment [15] que le trypanosome *T. mega*, cultivé *in vitro*, conserve indéfiniment l'aspect de crithidia et qu'il est possible d'obtenir expérimentalement la transformation des crithidias de culture en formes sanguicoles au moyen de sérum de divers vertébrés. L'exposé suivant tend à définir

quelques aspects de ce phénomène et montre notamment que la crithidia ne répond au *facteur transformant* du sérum qu'à un stade déterminé de sa croissance.

Nous avons emprunté au vocabulaire de l'embryologie causale le terme de *compétence* pour désigner la sensibilité d'une population de crithidias à l'agent morphogénétique.

MATÉRIEL ET MÉTHODES

Le trypanosome étudié est une souche de *T. mega* isolée par Boné [2] d'un crapaud, *Bufo regularis*, provenant du lac Tanganyka. Nous ne décrirons pas de façon détaillée les formes de culture et les formes sanguicoles de l'espèce *T. mega* utilisée dans nos recherches. Elles correspondent étroitement à l'aspect classique et suffisamment connu du genre. La forme sanguicole a fait l'objet d'études déjà anciennes de Dutton et Todd, de Broden et de Rodhain [9], tandis qu'aucune observation de la forme modanienne de ce parasite ne semble avoir été publiée jusqu'à présent.

La souche utilisée est cultivée *in vitro* depuis trois ans. Le milieu de culture contient dans un volume final de 1 litre, 4 g NaCl, 0.4 g KCl, 5 g $Na_3PO_412H_2O$, 2 g glucose, 15 g Bacto-Tryptose Difco et 1 g Oxoïd Liver Infusion. De l'hémine est ajoutée à raison de 20 mg par litre, sous forme d'une solution alcoolique alcalinisée (hémine 20 mg, EtOH 10 ml, NaOH 0,025 *M* 10 ml). Le pH est ajusté à 7,8 au moyen d'HCl normal. Le milieu est stérilisé à l'autoclave pendant 10 minutes à 10 lbs. La température de 26°C s'est montrée la plus favorable aux cultures.

De bonnes croissances des trypanosomes peuvent être obtenues sans agitation dans des boîtes de Roux ou des erlenmeyer. Une méthode meilleure est celle communément utilisée pour les cultures de tissus en tube à essai (roller tube). Pour les cultures massives du flagellé, nous avons trouvé l'emploi de bouteilles cylindriques en verre pyrex (serum bottles) particulièrement favorable. Chaque bouteille de 4 litres reçoit 0,5 litre de milieu de culture et est couchée sur un agitateur à rouleaux qui lui imprime une rotation régulière d'environ $\frac{1}{4}$ tour par minute.

Les essais de transformation se font dans des tubes contenant chacun 2 ml de la préparation de sérum et 1 ml de milieu de culture bacto-tryptose. Ces milieux transformants sont ensemencés d'environ 10^6 trypanosomes par millilitre. Le nombre initial de flagellés est compté immédiatement après l'inoculation, les trypanosomes transformés sont comptés 48 heures après.

Les comptages de trypanosomes sont effectués dans une cellule de Thoma, à l'aide d'une optique à contraste de phase.

Préparations diverses de sérum

Nous avons utilisé pour ces expériences du sérum de bœuf ou de veau. Les sérums utilisés entiers sont préalablement chauffés pendant 30 minutes à 56°C pour inactiver des lysines anti-trypanosome.

Sérum traité à la ribonucléase (RNase).—10 ml de sérum de veau (pH 8,0) sont incubés à 37° pendant 2 heures avec de la RNase cristallisée. L'enzyme est ajouté

au sérum à trois reprises, à 45 minutes d'intervalle, sous la forme d'une solution fraîchement préparée dans l'eau distillée. La quantité totale de RNase ajoutée aux 10 ml de sérum est de 2 mg.

Sérum traité à la désoxyribonucléase (DNase).—0,1 mg de DNase cristallisée et 0,1 ml d'une solution de chlorure de magnésium 0,3 *M* sont dissouts dans 10 ml de sérum de veau (pH 8,0). Ce sérum est incubé à 37° pendant 2 heures. Le sérum témoin est incubé de la même façon en présence de Mg^{++} mais sans DNase.

Après l'incubation à 37°, ces sérums traités à la RNase ou à la DNase sont chauffés pendant 30 minutes à 56°C.

Déprotéinisation du sérum.—Le sérum est dilué d'un égal volume d'eau distillée et le pH amené à 5,6 avec de l'acide chlorhydrique. Il est ensuite chauffé à 100°, au bain-marie, pendant trente minutes. Le précipité est éliminé par centrifugation. Le surnageant est ramené à pH 7,4 avec de la soude. Certaines expériences ont été faites avec une préparation semblable mais chauffée à pH 7,8 au lieu de 5,6. Dans ce cas une quantité importante de lipoprotéines reste soluble. — Les résultats obtenus avec cette seconde fraction sont identiques à ceux que l'on obtient avec la première.

Sérum chauffé à 100° et traité à la trypsine.—Le sérum déprotéinisé par la méthode décrite plus haut est incubé pendant 150 minutes à 37°C avec 0,2 mg/ml de trypsine cristallisée (NBC°). Cette préparation est ensuite stérilisée à l'autoclave, refroidie et additionnée de 0,4 mg d'ovomucoïde par millilitre. Dans ces conditions, l'ovomucoïde, préparé selon Lineweaver et Murray [10], bloque entièrement toute activité ultérieure de la trypsine. L'ovomucoïde n'exerce aucune action visible sur les trypanosomes.

L'azote total acido-insoluble d'une culture est déterminé par la technique de Kjeldahl sur les trypanosomes centrifugés et lavés à l'acide trichloracétique 5 %.

PARTIE EXPÉRIMENTALE

Développement de la forme crithidia en culture.—La croissance des crithidias dans le milieu bacto-tryptose est exponentielle pendant les 5 à 8 premiers jours après l'inoculation, selon la richesse de l'inoculum. La culture peut cependant se maintenir en vie dans le même milieu pendant plus de deux mois. Le temps moyen de génération est d'environ 24 heures. Pendant toute la phase de croissance logarithmique on assiste à une évolution très nette

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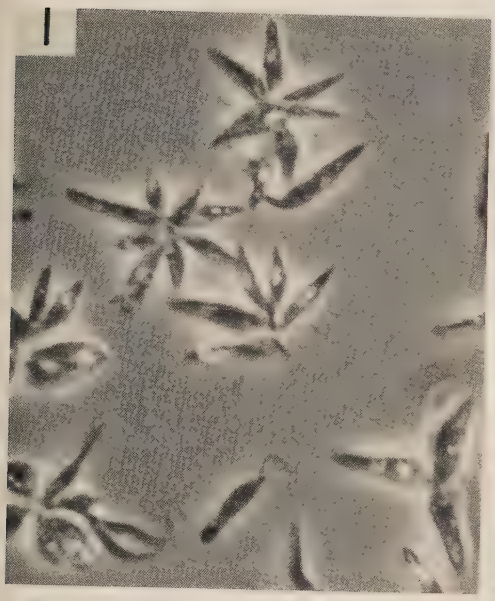
Fig. 1.—Rosaces de crithidias du type court dans une culture en croissance. Préparation vivante — contraste de phase.

Fig. 2.—Longues crithidias d'une culture arrivée au terme de sa croissance exponentielle. Préparation vivante — contraste de phase.

Fig. 3.—Crithidias semblables à celles de la figure 2, mais fixées et colorées au Giemsa. On remarque la position antérieure du kinétoplaste et l'absence de membrane ondulante. Grossissement : 950 ×.

Fig. 4.—Trypanosomes transformés, obtenus en inoculant des crithidias semblables à celles de la figure 3 dans un milieu contenant la fraction de sérum thermostable. *T*, Trypanosomes transformés; *C* Crithidias de type court, développées à partir d'individus non sensibles. Même grossissement que pour la figure 3.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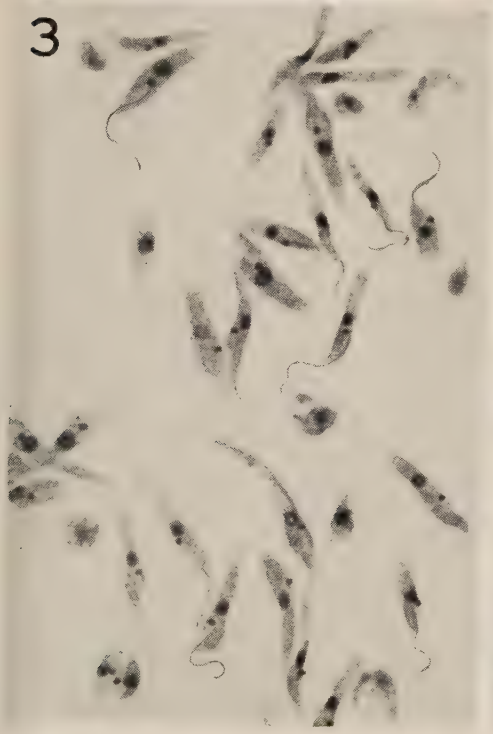
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du flagellé. Deux types morphologiques de la crithidia se succèdent : (1) la forme courte peu mobile (fig. 1), formant des rosaces de 2 à plusieurs dizaines d'individus, (2) la forme longue crithidia (fig. 2, 3), toujours libre et très mobile. Des stades intermédiaires existent mais sont éphémères. Il n'y a pas de membrane ondulante chez ces deux types de crithidia et le kinétoplaste est toujours antérieur par rapport au noyau. Ces caractères les différencient nettement de la forme trypanosome (fig. 4).

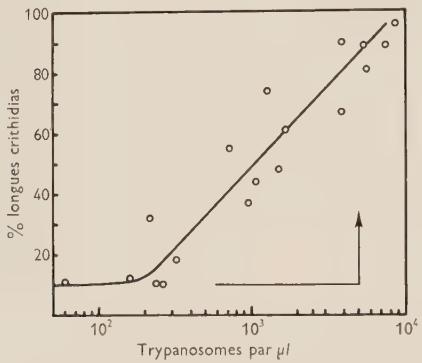

Fig. 5

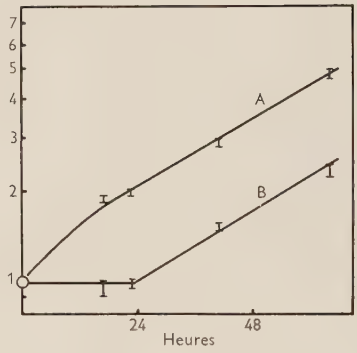

Fig. 6

Fig. 5.—Variation du pourcentage de longues crithidias au cours de la croissance de la culture. Résultats réunis de plusieurs expériences. En abscisses : le nombre total d'individus par μl. La flèche indique la fin de la croissance logarithmique.

Fig. 6.—Phases initiales de la croissance et de la multiplication des crithidias en culture. *Ordonnées* (valeurs relatives) : *A*, Azote total précipitable par l'acide trichloracétique; *B*, Nombre d'individus par unité de volume. *Abscisses* : Temps à partir de l'inoculation.

Les générations de la phase initiale de la croissance exponentielle sont toutes du type court en rosaces. Lorsque la densité de la population atteint environ 2000 individus par mm^3, les rosaces commencent à se défaire et libèrent un nombre toujours croissant de crithidias longues (fig. 5). La proportion de ces formes libres atteint 90 à 100 % à la fin de la phase de croissance logarithmique. La succession de ces deux types morphologiques se manifeste parfois par une inflexion de la courbe de croissance (exprimée en poids total d'azote protéique) (fig. 8).

Dans une culture fraîchement inoculée, les premières divisions sont précédées d'abondantes synthèses azotées (fig. 6). La teneur individuelle en azote acido-insoluble est maximale dans les crithidias prelevées au début de la phase de multiplication (fig. 7); cette teneur en azote décroit ensuite pour tomber à la fin de la phase logarithmique au quart de la valeur maximale.

Transformation expérimentale de la crithidia.—Inoculées dans un milieu contenant du sérum, les crithidias de culture (fig. 3) peuvent se transformer et acquérir la forme trypanosome (fig. 4). Le corps du flagellé s'allonge d'abord en s'amincissant, l'extrémité postérieure, obtuse chez la crithidia, devient très effilée. Le kinétoplaste se déplace en arrière du noyau en même

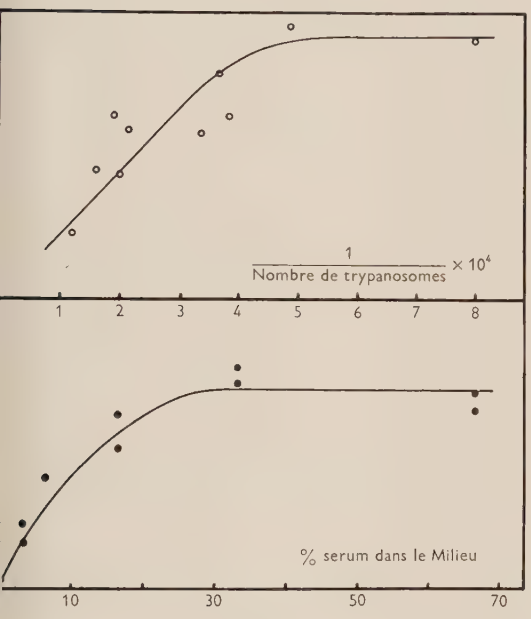

Fig. 8

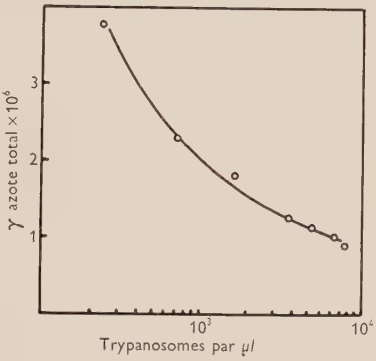

Fig. 7

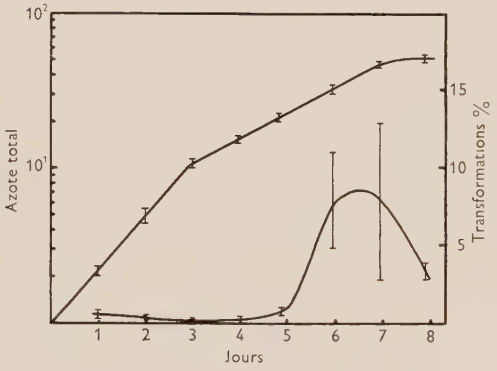

Fig. 9

Fig. 7.—Variation de la teneur individuelle des crithidias en azote précipitable par l'acide trichloracétique, en fonction de l'âge de la culture. En abscisses, le nombre total d'individus par μl.

Fig. 8.—Variations du pourcentage de transformations en fonction du nombre de crithidias par unité de volume et de la richesse du milieu en sérum. *Courbe supérieure* : expérience réalisée en inoculant des quantités variables de crithidias dans un milieu contenant 66 % de sérum. En abscisses : inverse du nombre de trypanosomes par μl de milieu. *Courbe inférieure* : expérience réalisée en inoculant un nombre constant de crithidias (1300/μl) dans des milieux de teneurs croissantes en sérum.

Fig. 9.—La compétence d'une culture de trypanosomes en fonction de sa croissance. *Courbe supérieure* : la croissance de la population dans le milieu Bacto-tryptose, exprimée par la teneur totale en azote précipitable par l'acide trichloracétique (valeurs relatives). *Courbe inférieure* : La compétence de cette même population, exprimée par le pourcentage d'individus qui se transforment lorsqu'ils sont transférés dans un milieu contenant 66 % de sérum.

temps qu'apparaît la membrane ondulante. Enfin la taille du trypanosome augmente et atteind des dimensions très supérieures à celles de la crithidia.

Compétence.—Le nombre de crithidias qui se transforment en trypanosome dépend de la quantité de sérum mis à leur disposition: lorsqu'on inocule un nombre N de crithidias d'une culture dans un milieu contenant une certaine quantité S de sérum, on observe que le nombre de transformations est d'autant plus élevé que le rapport S/N est grand. La fig. 8 illustre ce phénomène et montre également que pour une certaine valeur du rapport S/N, le pourcentage de transformations atteint un palier qu'il n'est pas possible de dépasser. Il faut donc admettre que la population de crithidias contient des cellules sensibles et d'autres non sensibles à l'agent morphogénétique du sérum. Le pourcentage ce cellules sensibles représente ce que nous appellerons la compétence de cette population.

La compétence est étroitement liée à l'âge de la culture, comme le montre la fig. 9. Au début de la croissance logarithmique la proportion de crithidias sensibles à l'agent morphogénétique est très faible, toujours inférieure à 1 %. La compétence de la culture augmente vers la fin de la phase exponentielle, atteint rapidement un maximum et diminue ensuite. La compétence maximale précède immédiatement le point d'inflexion de la courbe de croissance ; à ce moment le nombre de crithidias sensibles est de l'ordre de 10 % ou plus. Une expérience nous a donné près de 50 % de transformations, mais le fait semble exceptionnel. Les crithidias non sensibles se comportent comme dans le milieu de culture sans sérum. Elles reviennent rapidement au type court et se divisent en formant des rosaces (fig. 4).

La prolifération rapide des crithidias non sensibles empêche une observation prolongée des trypanosomes transformés. Cependant, au cours des cinq ou six jours durant lesquels cette observation est possible, nous n'avons jamais observé la multiplication de ces flagellés de type sanguicole mais leur taille s'accroît dans de fortes proportions.

Facteur transformant. Le facteur transformant fut mis en évidence dans le sérum de divers vertébrés (crapaud, souris, cheval, bœuf, porc). Un facteur analogue se trouve dans la cervelle de bœuf et dans le jaune d'œuf de poule (extrait à 100° dans tampon phosphate pH 7). Le sérum déprotéinisé par chauffage à 100° à pH5,6 conserve toute son activité morphogénétique. Cette préparation ne contient plus de substance précipitable par l'acide trichloracétique 10 %. Sa teneur en azote n'est plus que de 3 % de celle du sérum entier. La digestion prolongée par la trypsine, la ribonucléase ou la désoxyribonucléase n'altère pas la propriéte transformante du sérum.[1]

[1] La nature du facteur transformant a été determinée. Voir Exptl. Cell Research **15**, 431 (1958).

DISCUSSION

La culture de la forme sanguicole des trypanosomes, essayée depuis un demi-siècle, n'a jamais été réussie et, bien que la plupart des milieux de culture utilisés jusqu'à présent contenaient du sang défribriné ou du sérum, les formes sanguicoles ont rarement été observées en culture et toujours de façon peu reproductible et sporadique. Une des causes de cet échec nous paraît être la très faible compétence des crithidias au cours de la phase initiale de croissance. En effet, outre la présence du facteur sérique, les deux conditions suivantes sont nécessaires à l'apparition des formes sanguicoles en culture : (1) la population doit être numériquement faible, (2) elle doit comporter un pourcentage suffisamment élevé d'individus sensibles. Si la seconde condition n'est pas observée au moment de l'inoculation, la compétence n'apparaît que lorsque la croissance logarithmique prend fin et à ce stade la densité de population est telle que la première condition n'est plus réalisée. De plus, même dans les cas ou les deux conditions sont réalisées simultanément lors de l'inoculation, on ne peut observer les formes sanguicoles que pendant la phase initiale de croissance de la population, car elles se perdent plus tard dans la grosse masse de crithidias qui se développent à partir des individus non sensibles.

On peut distinguer deux phases dans la morphogénèse de *T. mega* : l'évolution des crithidias dans une culture et la transformation de ces crithidias en trypanosomes. La première phase coïncide avec la période de croissance de la culture et se caractérise par un brusque accroissement de masse de la crithidia, suivi d'une décroissance progressive qui se prolonge pendant toute la phase exponentielle. Des phénomènes identiques ont été parfaitement étudiés chez des bactéries par Clark et Ruehl [5], Henrici [6], Hershey [7], et chez d'autres protistes par Scherbaum [12]. Ces résultats appuient l'hypothèse soutenue notamment par Swann [15] et par Scherbaum [13], que l'accroissement de la masse cytoplasmique et la multiplication sont des processus plus ou moins simultanés mais indépendants. Nos observations et celles, plus anciennes, de Chang (sur *Tr. cruzi* [4]) montrent que, chez le trypanosome, ces variations de la taille s'accompagnent en outre d'une évolution morphologique : crithidia courte–crithidia longue.

La seconde phase de la morphogénèse du trypanosome, c'est à dire la transformation de la crithidia en forme trypanosome, dépend de deux facteurs : (1) un facteur endogène correspondant à l'état physiologique particulier de la cellule sensible, (2) un facteur exogène propre à certains milieux biologiques (sérum). Ce second facteur n'est pas détruit par la désoxyri-

bonucléase comme le principe transformant du pneumocoque [11], ni par la ribonucléase. Il est peu probable d'autre part qu'il soit de nature protéique. Nous avions fait état, dans une publication préliminaire [14], de ce que l'activité transformante se trouvait liée aux globulines sériques précipitées par le sulfate d'ammonium. L'activité spécifique de ces préparations s'est cependant révélée très inférieure à celle du sérum total et pourrait être attribuée à une adsorption ou à une liaison labile du facteur transformant.

La fréquence des cellules sensibles varie de façon très nette au cours de la croissance de la culture. Pratiquement nulle pendant la phase d'accroissement exponentiel, la compétence de la culture augmente brusquement et atteint un maximum qui coïncide approximativement avec le point d'inflexion de la courbe de croissance ; elle diminue ensuite au cours de la phase stationnaire. La relation de cette sensiblité de la crithidia avec la croissance de la population dans un milieu limité ne manque pas d'analogie avec un aspect du développement des métazoaires, où le potentiel morphogénétique d'un territoire de la gastrula ne s'exprime qu'après une phase de segmentation obligatoire. Enfin, on est en droit de se demander si la compétence de la culture de trypanosomes n'est pas liée à une rupture d'équilibre dans certains mécanismes de synthèse, à une disharmonie de croissance (*unbalanced growth*) assez semblable à celle qui fut étudiée chez *E. coli* par Barner et Cohen 1954 [1]. La sensibilité des crithidias à l'agent morphogénétique apparaît en effet à un moment ou l'épuisement probable du milieu en certain(s) facteur(s) de croissance ou metabolite créerait cette rupture d'équilibre.

RÉSUMÉ

Trypanosoma mega cultivé *in vitro* dans un milieu dépourvu de sérum se multiplie indéfiniment sous la forme crithidia. Il existe dans le sérum des vertébrés un facteur capable de transformer certaines crithidias sensibles en formes trypanosomes.

L'apparition de la sensibilité à ce facteur morphogénétique est conditionnée par la croissance de la population de crithidias.

Nous remercions très vivement le Docteur G. J Boné, Chef de Centre de l'IRSAC à Elisabethville, pour l'aide qu'il nous a souvent apportée dans ce travail Nous tenons aussi à exprimer notre reconnaissance envers MM. les Professeurs R. Jeener, J. Brachet et le Docteur M. Errera pour leurs conseils, leurs encouragements et l'hospitalité qu'ils m'ont accordée dans leurs laboratoires de l'Université Libre de Bruxelles, où une partie de ces recherches a été effectuée.

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PRESSURE-CENTRIFUGE STUDIES ON THE MAST CELLS OF PERITONEAL FLUID IN RATS OF VARIOUS AGES[1]

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The reversible solation and gelation of protoplasm has been investigated in diverse cells and apparently may play a major role in converting metabolic energy into mechanical work. In studies concerned with sol↔gel equilibria, investigators have employed as a frame of reference, form and locomotion in ameboid organisms [13, 24], cleavage in various marine eggs [1, 18, 33, 34, 36], cyclosis in plant cells [19, 20], cardiac rate in explanted embryonic frog heart [11], and Brownian movement [8]. To our knowledge, little or no work has been done on sol-gel changes in mammalian cells.

The biophysics of cellular shape has been discussed by Lewis [16] and others [5, 35]. It is generally agreed, on the basis of established physico-chemical principles, that cells freely suspended in a fluid medium will tend to assume a perfectly spheroid form,[4] a condition satisfied under both physiological conditions (leukocytes carried by circulating blood and other cellular types in various exudates) and nonphysiological conditions (tissue culture of the continuous agitation type [26]). From such physico-chemical considerations, it follows that in the solated state, cells are forced to assume a spherical shape, just as fluid droplets will when dispersed in an immiscible solvent. Similarly, departure from the spherical shape must be dependent on the expenditure of energy to achieve cytoplasmic plasticity (gelation). A consideration of gel types as discussed by Freundlich [4] has been extended to biological systems [23].

The cellular types encountered in peritoneal fluid of intact rats are eosinophilic leukocytes, various monocytic elements (lymphocytic and macropha-

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[4] It is assumed here that the cell in question does not have any form of "skeleton" or supporting structure such as crystalloid, spicules, or cuticle.

gic cells), and mast cells [28]. In immature or young adult animals, all these cells are visualized as perfectly spherical when the peritoneal fluid is first observed in the hemacytometer immediately upon removal from the peritoneal cavity. After settling to the floor of the counting chamber, the mast cells remain apparently unchanged.

In light of the above considerations, the observation that many mast cells at rat periteoneal fluid are not spherical in hypophysectomized [32] or in older [30] animals assumes particular interest. It has been suggested that aging is characterized by progressive dehydration of the cell (even in the face of *tissue* hydration) [3] perhaps accompanied by changes in intracellular calcium [9, 14, 15]. Such changes would presumably lead to increased cytoplasmic gelation. The mast cells of rat peritoneal fluid therefore appear especially well suited to the study of cytoplasmic sol-gel equilibria for comparison to cells of other phyla. This paper is concerned with some quantitative aspects of cytoplasmic gelation in mast cells of the rat. In addition, changes in this parameter have been studied with particular attention to aging. A preliminary note on this work has been published [37].

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain, 90–100 g in body weight (approximately one month old) were obtained from a commercial source[1] which also supplied their birth date. The animals were housed in standard wire mesh bottom laboratory cages and received food[2] and water *ad libitum* until sacrificed.

The apparatus employed has been described in detail elsewhere [1, 21]. A rat was anesthetized lightly with ether and exsanguinated by decapitation. Peritoneal fluid was collected as previously described [31] and transferred into a siliconed[3] vial which was then sealed with parafilm and placed in a refrigerator at 14°C until needed. Meanwhile, the pressure centrifuge housing was equilibrated at 20°C. A small sample of peritoneal fluid was drawn into a capillary pipette, transferred to the pressure-centrifuge tube and overlaid with 0.9 per cent saline at 20°C. The required pressure was rapidly established, the experimental tube was sealed by the needle valve and the centrifuge head transferred to the high speed centrifuge and spun at 41,000 × *g* for the required time. Speed of revolution was controlled by regulating the voltage input to the centrifuge motor, and was standardized with a stroboscopic lamp. After centrifuging, the pressure was released, the cells were withdrawn with a capillary pipette, placed into a hemacytometer (Spencer bright line) and rapidly enumerated at a magnification of 300 × (Zeiss Lumipan binocular microscope, 20 × objective and 10 × oculars). The percentage of deformed mast cells was rapidly determined

[1] The Holtzman Co., Madison, Wisc.
[2] Purina pellets, The Ralston Cereal Co., St. Louis, Mo.
[3] Siliclad, Clay Adams Co., N.Y.

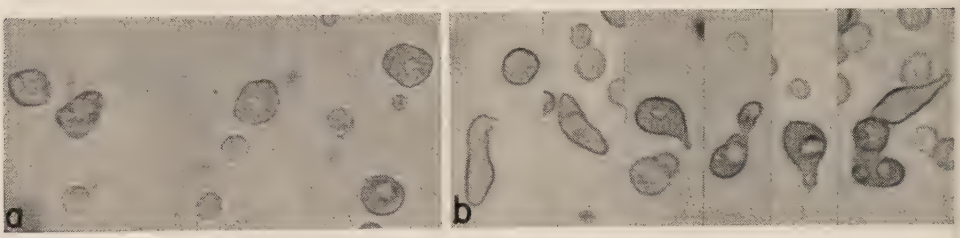

Fig. 1.—Peritoneal mast cells of the rat as seen in hemacytometer. Physiological saline diluent. (*a*) Five undeformed cells from aliquot spun for 120 seconds at atmospheric pressure. (*b*) Varying degrees of deformation as seen in aliquot spun 12 seconds under hydrostatic pressure of 13,000 lbs./in^2. This forms the basis for the quantitative measurement of gelation.

for a minimum of 150 cells; more frequently between 200 and 300 cells were enumerated with two Veeder-Root counters, one kept in sight (total count) and the other out of sight (deformed cells count). The point at which a cell was considered to be deformed is admittedly a subjective one and required standardization. In order to reduce variations, all counts were performed by a single observer (J. P.). As controls, a number of determinations was carried out on material whose centrifugation time and pressure history were decided upon and recorded by one experimenter (A. M. Z. or W. A.), but unknown to the sampler (J. P.). Normal cells, and cells in varying degrees of deformation are illustrated in Fig. 1.

The experiments described here cover a span of approximately 18 months and were actually performed at two different periods one year apart. The animals were divided into three groups and sampled as described in Table I. It should be noted that the oldest animals (59 wks) belonged to a group of which a part had been sampled the previous year and also that a common age group (approximately 20 wks) was utilized as control in each experimental period.

For each pressure, repeated aliquots of a peritoneal fluid sample were centrifuged until the period of time required to yield 25 $\pm$ 5 per cent deformed cells was established; an attempt was made to obtain values on both sides of the critical conditions. A line fitting the data best for each rat was drawn by eye and the points on the curve were read for pressures of 9000, 11,000, and 13,000 lbs/in.^{2}, and used subsequently for the statistical analysis. Technical difficulties (apparatus failure or insufficient amount of peritoneal fluid available to complete the experiment) and an appreciable death rate in the older group led to the rejection of numerous animals. Most of these fragmentary data, however, were in substantial agreement with those included herein.

TABLE I. *Experimental design.*

Gp.	Age when tested (date of testing; number of animals retained in group)	
I	7 wks (7/56; 4)	
II	20–23 wks (7 & 8/56; 6)	59 wks (7/57; 3)
III	19–21 wks (7/57; 6)	

RESULTS

Under certain conditions, mast cells were deformed in the pressure-centrifuge, the relative numbers of cells affected depending on the pressure and centrifugal forces used. Deformation consisted essentially of an elongation of the cell with little, if any, sedimentation of the cytoplasmic granules (Fig. 1). With sufficiently high centrifugal forces and pressures, the mast cells eventually fragment. For the quantitative assays described below, samples were discarded whenever fragmentation was evidenced. The duration of centrifugation required to deform 25 ± 5 per cent of the mast cells is dependent on the pressure maintained in the system over the range of 7000 to 14,000 lbs/in.^{2} tested. Below 7000 lbs/in.^{2}, centrifugation time became prohibitively long

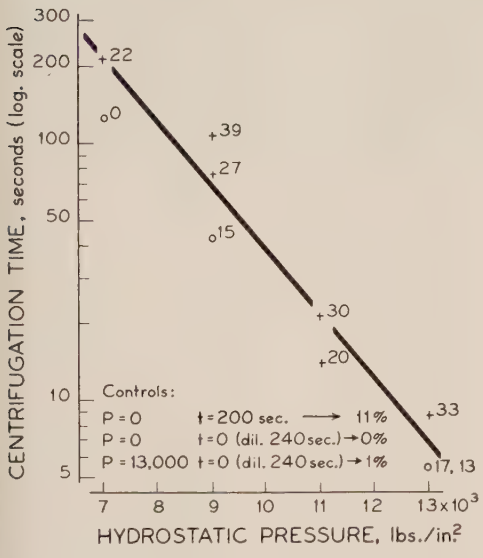

Fig. 2

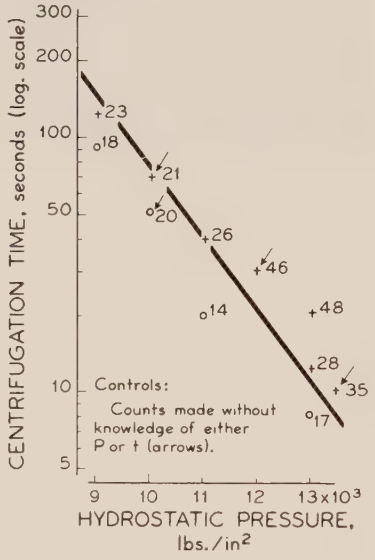

Fig. 3

Fig. 2.—Relative gel strength (ordinate) of mast cells as a function of pressure. Conditions causing 25 ± 5 per cent deformed cells denoted by +, less than this value by 0. Accompanying numbers represent percentages of deformed cells encountered. Note that for any given pressure, the percentage of deformed cells increases as centrifugation is prolonged. Curve drawn by eye. Representative example. (Rat #24, born Feb. 20, 1957; tested July 15, 1957, 21 weeks old.)

Fig. 3.—Same as Fig. 2, but controls designed to test for unconscious bias. Here again, note that increasing centrifugation time at any given pressure leads to progressively greater percentages of deformed cells. It can be seen that the control values (arrows) fell in expected relationship to the curve previously determined for this particular animal. All "blind" determinations were completed before ascertaining whether control was meeting with success or failure, so that no readjustment was possible between counts. (Rat #19, born Feb. 20, 1957; tested July 5, 1957, 19 weeks old.)

in most cases and good temperature control could not be maintained; with pressures greater than 14,000 lbs/in.^{2}, the required centrifugation time became very short and large timing errors were introduced. The control procedures employed, including (1) dilution and handling of the cells, (2) centrifugation at 41,000$\times g$ and atmospheric pressure, and (3) pressurization without centrifugation all proved to have only minor effects on mast cell morphology (Fig. 1*a*, Fig. 2). At any given pressure, small variations in centrifugation time resulted in correspondingly graded responses when based on the percentage of cells affected (Figs. 2, 3). The *extent* of deformation, however, appeared to be poorly correlated to the applied force. These observations are illustrated in Fig. 2, where it can be seen that an inverse linear function obtains between the logarithm of the centrifugation time (relative gel strength) and the pressure. Additional controls, designed to test for unconscious bias in determining the percentage of deformed cells (Fig. 3) showed our techniques were essentially reliable.

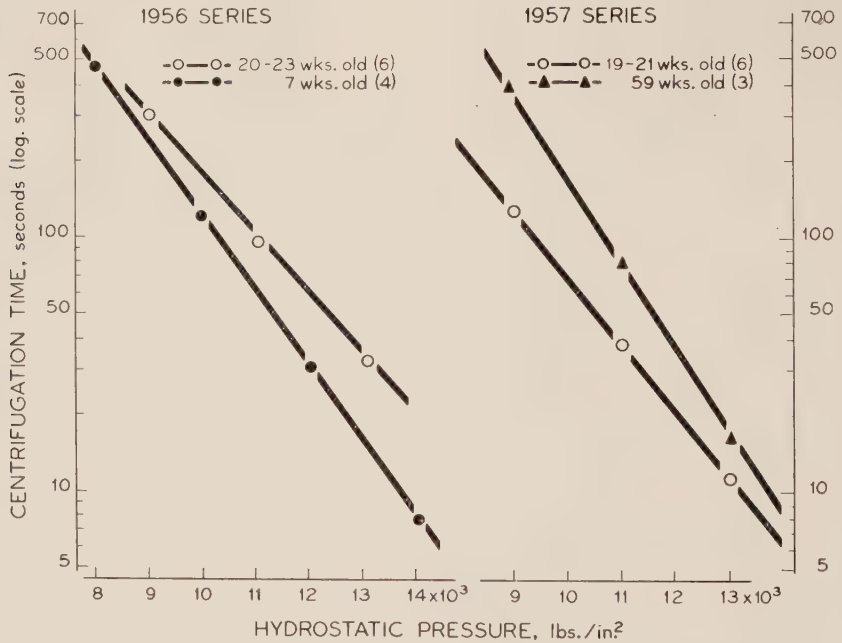

Fig. 4

Fig. 5

Figs. 4–5.—*Fig. 4 (left):* Relative gel strength of mast cells in 7 and in 20 week old rats. Curves based on averages for each group. Experimental series of 1956. *Fig. 5. (right):* Relative gel strength of mast cells in 20 and 59 week old rats. Curves based on averages for each group. Experimental series of 1957.

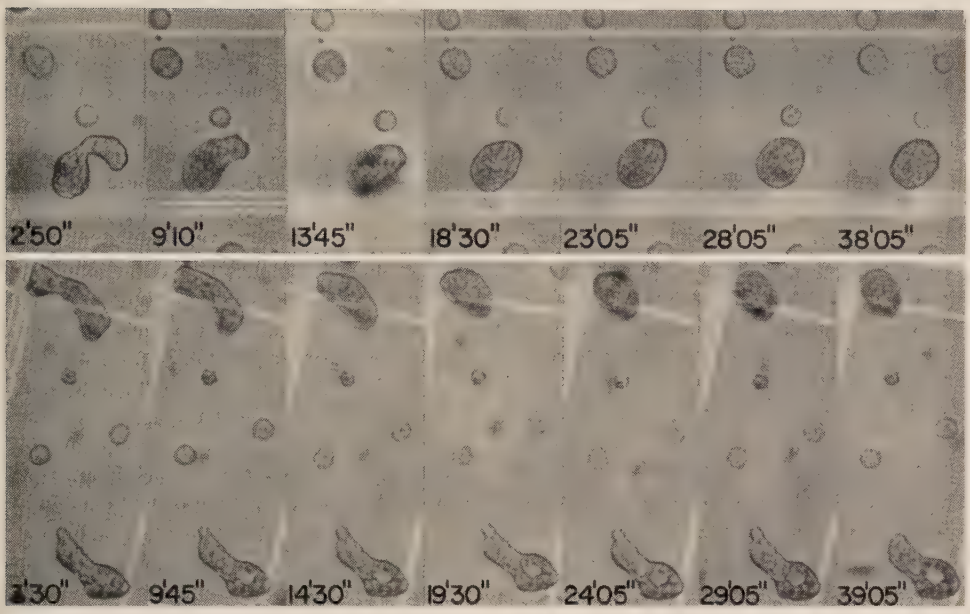

Fig. 6.—Reversal, at room temperature and atmospheric pressure, of deformation effected in the pressure-centrifuge. Vertical row on left: cells as observed when first withdrawn from experimental chamber. Horizontal rows represent various stages of recovery; rate can be ascertained by reference to time record started at end of spin. In upper row, mast cell would have been considered undeformed if seen at 14 min. stage; complete reversal not reached until 28 min. stage. A similar situation prevails for upper cell of lower row, but note that cell at bottom of photomicrograph begins to alter morphologically only after a lag of approximately 30 min.

On the average, the cytoplasmic gel strength of mast cells from peritoneal fluid of 7 week old rats was lower than corresponding values for 20 week old animals (Fig. 4). In a similar set of experiments, a second group of 20 week old rats yielded appreciably lower absolute values than those obtained the previous year for a similar group, but these appeared to be lower than corresponding data for 59 week old animals (Fig. 5).

The deformation of mast cells in the pressure-centrifuge is a reversible process: deformed mast elements[1] allowed to stand in the hemacytometer at room temperature (24–25°C) revert to a spherical shape within 20 45 minutes (Fig. 6). Deformation of non-mast elements was noticed to parallel changes in mast cell morphology; these were not quantitated, however, because of technical difficulties attending their smaller size.

[1] Only mast cells obtained from 20 week old group were quantitated in this respect. It may well be that the rate of recovery is an age-dependent variable; experiments to test this hypothesis are contemplated.

DISCUSSION

An extensive literature has accumulated dealing with some physical parameters of living cells, including tension at the surface, elasticity of the membrane, cytoplasmic viscosity and sol-gel transformations. Whereas these various properties must ultimately be dependent on the state of the various constituent molecular species of the system, that is at the chemical or biochemical level, much can be adduced by purely physical measurements. To the investigator who has not employed physical techniques, these often appear to be unusually harsh, particularly where whole intact cells are subjected to centrifugation at thousands of times gravity or to hydrostatic pressures up to 15,000 lbs/in.^{2}. Actually, cells are surprisingly resilient to many physical factors, although rather sensitive to others such as temperature. For instance, in the classical experiments of E. B. Harvey reviewed recently [7], centrifugation of marine eggs sufficient to cause stratification and even fragmentation nevertheless did not interfere with the ability of the merogones to undergo early cleavage. Similarly, cleavage in various eggs and ameboid motion in various Sarcodina can resume normally following repeated exposure to high hydrostatic pressures [2, 13, 22, 24].

As already mentioned above, a *solated* cell freely suspended in a fluid medium would assume a spherical shape because this condition, from physico chemical considerations, satisfies the requisite that an equilibrium state of minimum energy be reached, as indeed it is when the cell surface-to-volume ratio is at a minimum. It must be emphasized, however, that whereas this argument holds for solated cells under specified conditions, the converse need not hold: all spherical cells in a fluid medium are not necessarily in the sol state even though non-spherical cells must necessarily be appreciably gelated. In addition, whereas protoplasmic gelation is an endothermic process (type II of the Freundlich classification), and whereas a gel is formed from solated protoplasm only if energy is expended, the maintenance of the gel structure need not require energy. For a further consideration of hydrostatic pressure in biological systems, the reader is referred to Johnson, Eyring and Polissar [10].

In the experiments described here, we are measuring the amount of work (centrifugation force $\times$ centrifugation time) that must be done on the cell to effect deformation when the cell is pushed against the bottom of the centrifuge chamber under various degrees of protoplasmic gelation. It is apparent that we are using an end point encompassing a number of variables, such as surface tension (including elastic components) and internal gelation of the cell.

For this reason, our data are not strictly comparable to those obtained in studies on ameboid motion or cytokinesis and may well account for the relatively higher pressures required to affect mast cells. Yet, it can be seen from Figs. 2 and 3 that the slope of the curve differs but slightly from those obtained for other systems where the end point could be defined more narrowly, primarily in terms of a gelation component; whereas the slope of curves describing the marine eggs and ameba data is approximately 2.5×10^{-4}, it ranges from about 2.9×10^{-4} (7 week old rats) to 2.3×10^{-4} (20 week old rats) in the present experiments. These values are in surprisingly good agreement with other gel strength studies already mentioned.

It is of interest that deformation of mast cells by the means employed in the present experiments is readily reversible. No lysis takes place and the reversal is indeed complete for the entire population under observation. This seems to argue that the observed cells are essentially normal even at the time when the measurements are obtained.

For both periods when the studies were carried out, animals of approximately 20 weeks of age were tested (group II in 1956 and group III in 1957), thus affording a common reference standard. On an absolute basis, these differed appreciably, but it should be noted that the two curves have essentially the same slope (compare upper curve of Fig. 4 and lower curve of Fig. 5). The apparent discrepancy between the two years is obviously due to a difference in what was accepted as effective deformation. Such an end point must be established arbitrarily and is valid only if proven reliable and reproducible. That this was indeed the case is evidenced by the high predictability of the data during each run as well as by the identical slopes obtained. It is thus clear that only relative values have significance and it is therefore permissible to compare all the data obtained once they are recalculated on a common basis. This has been done by arbitrarily setting as unity the average value at 13,000 lbs/in.^{2} for the common age groups (20 week old) and calculating all other values within the group as well as those in their respective experimental group, relative to this point. In doing so, the two reference curves become superimposable within the experimental error (Fig. 7). It is apparent that peritoneal mast cells from seven week old rats are generally more easily deformed (lower gel strength), while those from fifty nine week old animals are less so (greater gel strength), than those obtained from young adult donors (20 week old) over the range of pressures utilized. Whether this progressive increase in cytoplasmic gelation as a function of aging can be assumed to occur under physiological conditions (atmospheric pressure, 14.7 lbs/in.^{2}) is not clear. With other test systems, in which the nature of the experimental

material has allowed measurements to be obtained down to atmospheric pressures, the slope of the log.-centrifugation time versus pressure curve has remained constant [12, 13, 19, 36]. The validity of extending these findings to mammalian cells has not yet been established, however, and extensive extrapolation of the present data from 8,000 lbs/in.^{2} to atmospheric pressure is not justified, particularly since the end point used here—deformation of

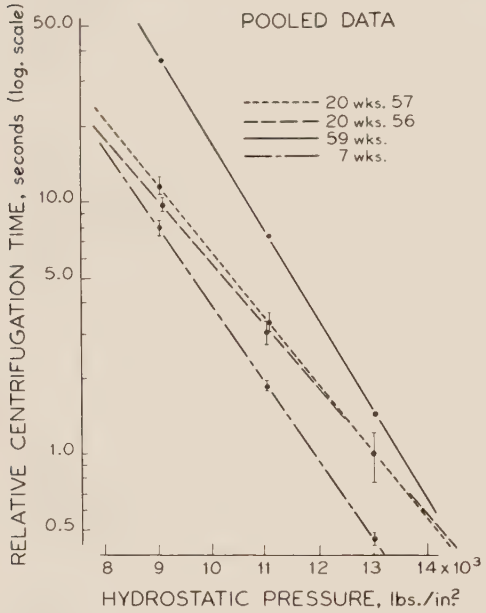

Fig. 7.—Cytoplasmic gelation of mast cells recalculated relative to $P_{13,000}^{20\ wks.}$. Curves based on averages for each group, with vertical brackets representing Standard Error of the Mean. It can be seen that, with increasing age, the curve is displaced upward, indicating increased gel strength.

the cell—is not the same as that used in cytokinesis or ameboid motion experiments (see above). Nevertheless, should a linear extrapolation to atmospheric pressure be compatible with the facts, and proof of such a hypothesis is dependent on further technological advances, the relative cytoplasmic gel strength values between mast elements of young and mature rats under physiological pressures would obviously differ from that demonstrated above 8000 lbs/in.^{2}. Too few animals survived in the 59 week old group to allow more than a qualitative consideration of the upper curve of Fig. 7. It should be noted that complete solation of the cell was never accomplished in our experiments since no change of slope occurred even at the highest pressures used.

Peritoneal mast cell morphology has been studied as a function of aging and it has been shown that non-spherical cells are increasingly numerous as the donor animal gets older [30]. The mast cells found in peritoneal fluid apparently represent cells shed from the mesenteries [25], in which tissue

they often occur as aspherical entities. The reversibility of morphological alterations effected in the pressure-centrifuge allows us to postulate that "abnormal" shapes (that is, recently shed non-spherical cells) equilibrate so rapidly in young rats that they are rarely caught in a transition stage, while just the opposite may be true for older animals [30] and following hypophysectomy [32]. In 20 week old rats, such a morphological readjustment requires approximately 30 minutes at 23°C *in vitro* (Fig. 6), but it may occur faster at body temperature. It should be emphasized that the type of morphological "aberration" discussed here differs from that visualized following administration of colchicine [29] or various derivatives of colchicine and podophyllotoxin [27].

From our experiments, it appears that the changes in cytoplasmic gel strength accompanying aging of the animal are referable to most, if not to all the mast cells within the peritoneal cavity regardless of their initial shape. In other words, this is a *population* effect, suggesting that the controlling factor is referable to the *milieu intérieur* and is independent of the age of individual mast cells. This latter parameter cannot be determined at present since the very genesis of mast cells is still open to question; however, it is most unlikely that mast cells in any given population are all of the same age.

The thesis that calcium plays a role in determining gel strength and that it accumulates in aging organisms has been advanced primarily by Heilbrunn [9] and by Lansing [14, 15]. The significance of such arguments in relation to the rat are still open to question, however, since experimental determination of calcium in rat liver, brain and kidney do not show changes in the concentration of this metal as a function of aging [17]. A refinement of the argument, namely that aging may be associated with a redistribution of intracellular calcium, is not supported by work on liver cell fractions of young and old rats [6].

Our data are therefore compatible with the thesis that increased gelation is a feature of aging. It remains to be demonstrated whether experience with mast cells can be extended to other cellular types. It would also be important to investigate the properties of mast cells in hypophysectomized rats and to relate the effectiveness of growth hormone in reestablishing the normal picture [32] to its possible role in regulating protoplasmic sol-gel transformations.

SUMMARY

The cytoplasmic gel strength of peritoneal mast cells of the rat has been determined by the use of pressure-centrifuge techniques. Results obtained indicate that this mammalian cell conforms qualitatively, and in certain re-

spects quantitatively, closely to the behavior of previously studied, non-mammalian cells.

Differences in gel strength are demonstrable as a function of age; the significance of such changes has been discussed.

Reversibility of morphological alterations induced by pressure-centrifuge methods has been demonstrated and related to the normal occurrence of non-spherical peritoneal mast cells in older animals.

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THE ACTIVITY OF SOLUBLE CYTOPLASMIC CONSTITUENTS FROM REGENERATING RAT LIVER IN AMINO ACID INCORPORATING SYSTEMS

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It was shown in previous papers that cytoplasmic preparations from regenerating rat liver could incorporate labeled amino acids into proteins more actively than similar preparations from normal liver [17, 9]. The cytoplasmic systems which were used in these experiments contained soluble constituents of liver homogenates combined with microsomal material. As is well known, enzymes from the soluble fraction are needed in experiments of this kind for the initial activation of the labeled amino acids at the expense of high-energy phosphates. Microsomal components, on the other hand, are required for the subsequent introduction of the reactive amino acid residues into protein molecules [33, 13, 16]. It was observed that both the microsomes and the soluble fraction contributed to the higher efficiency of the preparations from regenerating livers in the incorporation. When the activity of the microsomal preparations was measured at different periods after partial hepatectomy, a fairly rapid increase was observed after an initial lag period of about 12–14 hours. The activation was correlated with a modified composition of the microsomal material, possibly due to a richer attachment of nucleoprotein granules to the endoplasmic membranes.

The present paper is concerned with the activity of the soluble cytoplasmic components after hepatectomy. It was observed that the activity of these components increased about simultaneously with the activity of the microsomes. When compared on a percentual basis, however, the rise in activity of the soluble system was less pronounced.

METHODS

Male albino rats weighing approximately 250 g were used. Each experiment usually included 2–3 control rats and 2–3 partially hepatectomized rats, from which the median liver lobe, the left lateral lobe and the posterior lobule of the right lateral lobe had been removed under ether anaesthesia. Before the preparation of the homo-

genates the rats had been kept without food for 15–20 hours [15]. In several experiments 2–3 groups of rats, which had been hepatectomized at different periods, were used simultaneously in combination with a common control group of normal animals.

At different periods after hepatectomy (9 72 hours) the rats were killed by decapitation. The livers were chilled with ice and thoroughly perfused *in situ* with cold 0.155 *M* KCl. They were taken out, rapidly weighed, and then minced and repeatedly washed in an ice-cold medium, which generally contained 0.25 *M* sucrose, 0.025 *M* KCl, 0.01 *M* $MgCl_2$, 0.035 *M* tris(hydroxymethyl)-aminomethane (tris), pH 7.8. (In experiments, in which the soluble fraction would be dialyzed, sucrose was excluded from the medium, while instead the concentration of KCl was increased to 0.1 *M*). The liver mince was homogenized in a glass homogenizer with the same medium and was centrifuged for 6 minutes at 12,000 × *g* (0°C). The middle portion of the supernatant was transferred to the tubes of a Spinco preparative ultracentrifuge. The samples were centrifuged for 50 minutes at 105,000 × *g* (rotor 40) or for 60 minutes at 79,000 × *g* (rotor 30).

Activity of soluble fractions in incorporation systems.—In these experiments the incubation samples usually contained 0.08 μM of ^{14}C-L-leucine (1.5 mc per *mM*, Radiochemical Centre, Amersham), 1 μM of adenosine triphosphate (ATP, Sigma), 10 μM of phosphoenolpyruvate (synthesized according to Baer [1]), 0.2 μM of guanosine triphosphate (GTP, Sigma) and 0.3 ml of microsomal suspensions from normal or regenerating livers, containing about 5 mg of protein (determined by the method of Lowry *et al.* [23] in the presence of small amounts of deoxycholate). The microsomes were preferably prepared from 3.5 per cent homogenates, and the sediments, after careful washing of the pellets, were resuspended in cold homogenization medium by means of a small, hand-operated glass-homogenizer. In a number of experiments pyruvate kinase and myokinase were added to the incubation samples in optimal amounts, as estimated by preliminary experiments with different enzyme concentrations (Table I. The lower incorporation at high enzyme concentrations may be due to slight impurities in the enzyme preparations). The enzymes were prepared from rabbit muscle. Pyruvate kinase was purified to the "crystallization" stage of Beisenherz *et al.* (cf. [4]). Myokinase was purified as described by Colowick and Kalckar (cf. [6]).

In each experiment increasing amounts of supernatant from normal and regenerating livers were added to parallel series of incubation samples (Fig. 1). The protein content of the added supernatant usually covered the range of 0.25–1.5 mg per sample. The samples were finally made up to equal volumes (1–1.5 ml) by means of homogenization medium. The pH was then about 7.6, and the magnesium concentration usually 0.006 *M*. The samples were in most cases incubated at 35°C for 15 minutes. They were afterwards treated with unlabeled L-leucine in a large excess. The protein contents of all samples were made equal by the addition of calculated amounts of supernatant. The proteins were precipitated with TCA, purified by the method of Schneider [29] and plated on planchettes with an area of 1 cm^2. The radioactivity was measured, and the specific activities were calculated for indefinite sample thickness on the basis of an empirical activity saturation curve.

Measurements of activating enzymes. -Soluble fractions prepared from 30 per cent liver homogenates were used in these experiments.

ATP-pyrophosphate exchange was measured in the following way (cf. [10, 13, 8]). The incubation mixture contained in a final volume of 1.2 ml 4 μM of ^{32}P-pyrophosphate (prepared by prolonged heating of ^{32}P-K_2HPO_4 at 325°C), 6 μM of ATP, 6 μM of $MgCl_2$, 60 μM of KF and 50 μM of tris buffer pH 7.8. By including different amounts of supernatant in the incubation samples, concentration series were obtained

TABLE I. *Incorporation of ^{14}C-L-leucine into protein by normal rat liver microsomes in the presence of limiting amounts of supernatant.*

Effects of added pyruvate kinase and myokinase. The samples contained 0.8 μM of ^{14}C-L-leucine (1.5 mc per mM), 10 μM of phosphoenolpyruvate, 1 μM of ATP, 0.2 μM of GTP, and microsomes (approximately 5 mg of protein). They were made up to 1.0 ml by means of homogenization medium (0.25 M sucrose, 0.025 M KCl, 0.01 M $MgCl_2$, 0.035 M tris buffer pH 7.8). After the incubation, the samples were adjusted to equal protein contents. The same enzyme preparations, in the indicated amounts, were used in the subsequent experiments.

Supernatant, mg protein	Enzyme preparation, mg protein		C.p.m. per cm²
	Pyruvate kinase	Myokinase	
0.24	—	—	156
	1.4	—	145
	—	0.2	162
	1.4	0.1	166
	0.7	0.2	264
	1.4	*0.2*	283
	2.8	0.4	227
0.45	—	—	234
	1.4	*0.2*	337
	2.8	0.4	255

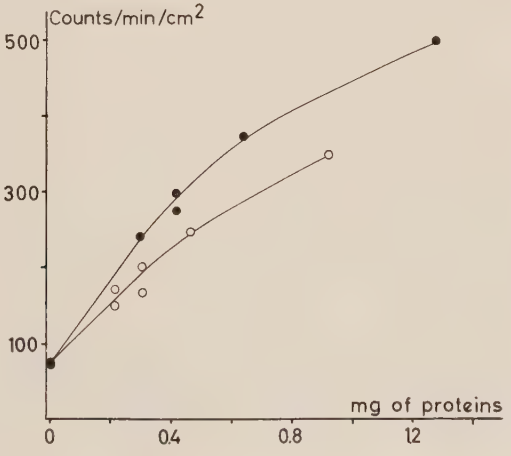

Fig. 1.—Incorporation of ^{14}C-L-leucine into protein by normal rat liver microsomes in the presence of varying amounts of soluble fractions from ((○) normal, and (●) regenerating (40 hours) liver. System as in Table I, including 1.4 mg of the pyruvate kinase and 0.2 mg of the myokinase preparations.

with protein concentrations within the range of 0.4–2.0 mg per sample. The protein concentration of the supernatants was determined by the method of Lowry *et al.* [23], crystallized bovine plasma albumin (Armour Lab.) being used as the protein standard. At each protein concentration duplicate samples without added amino acids were run in parallel with duplicate samples, to which 3 μM of each of the following amino acids (L-forms) had been added: alanine, arginine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, serine, threonine, tryptophane and valine. In some experiments a third set of duplicate samples was included, in which 3 μM of L-leucine had been added to each sample instead of the amino acid mixture. The samples were incubated at 35°C for 12 minutes and were then precipitated by the addition of 5 per cent trichloroacetic acid (TCA) to a final volume of 10 ml. After centrifugation 5 ml of the supernatant were treated with 200 mg of Norit for nucleotide adsorption according to the method of Crane and Lipmann [7]. The Norit was washed 3 times with water and was then treated for 15 minutes with N HCl at 100°C for the purpose of releasing the terminal ATP-phosphates. Samples of the total TCA-extract and of the Norit-supernatant were hydrolyzed in the same way. Phosphate determinations (by the method of Fiske and SubbaRow [12]) and radioactivity determinations were performed on duplicate samples from all of these three phosphate fractions. As a control of the efficiency of the adsorption process in separating pyrophosphate and ATP, samples incubated without enzyme were included in all experiments. The percentual exchange values were finally calculated by the equation of Davie *et al* [8].

Formation of hydroxamates was measured by the method of Hoagland *et al.* [13]. As amino acid substrate the same mixture of amino acids was used as in the exchange experiments.

Determination of ribonucleic acid.—The content of ribonucleic acid (RNA) in the supernatants was determined by the method of Ceriotti [5].

Measurement of pyruvate kinase activities. —The incubation system contained 3 μM of phosphoenolpyruvate, 3 μM of adenosine diphosphate (ADP), and varied amounts of supernatant. The final volume was adjusted to 1.0 ml by the addition of homogenization medium. The samples were incubated for 15 minutes at 35°C and were then precipitated with TCA. The amounts of pyruvate released by the reaction was measured by the 2,4-dinitrophenylhydrazine method of Lu [24] in the modification of Elgart and Nelson [11]. Controls without ADP were included in all experiments.

The activity of the reaction at different substrate concentrations is illustrated by Fig. 2. The reaction became progressively inhibited, when the concentration of ADP was disproportionately high in relation to the enzyme concentration. When the concentration of supernatant was varied at constant substrate concentrations, the curves obtained therefore became slightly S-shaped (Fig. 4). For the determination of enzyme activities only the concentration range giving perfectly linear curves was used. A pH-optimum of about 6.6 was observed (Fig. 3). Since we were especially interested in the activity of the enzyme under the conditions of the incorporation experiments, the activity in the supernatants was measured at that pH also. At pH 6.6 phosphate buffer was used instead of tris.

Measurement of adenylate kinase activities. Varied amounts of the supernatants (25–140 μg of protein) were incubated for 4 minutes at 25°C with a reaction mixture containing, per ml, 2 μM of ADP, 6 μM of $MgCl_2$ and 75 μM of tris buffer (pH 7.6, cf. [18]). The ATP formed by the reaction was determined by two separate methods.

(1) By the method used by Bowen and Kerwin [3] in myokinase determinations. In this case 80 μM of ethylenediaminetetraacetate (EDTA) were added to the incubated samples (1 ml) together with appropriate amounts of myosin B. The samples were again incubated at 25°C for release of ATP-phosphate, this time for 10 minutes. The phosphate was extracted with TCA and determined by the method of Fiske and SubbaRow [12]. Non-preincubated controls were run in parallel. Myosin B was pre-

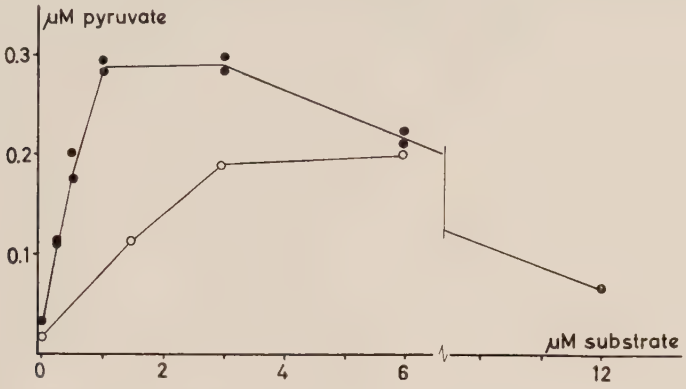

Fig. 2.—Pyruvate kinase from soluble liver fractions. Activity measured under standard conditions but at varied concentrations of (○) phosphoenolpyruvate, and (●) ADP.

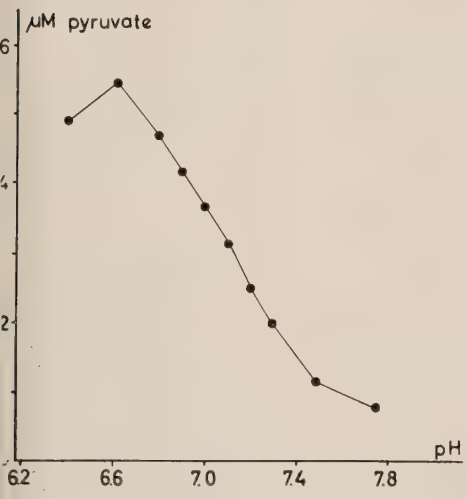

Fig. 3

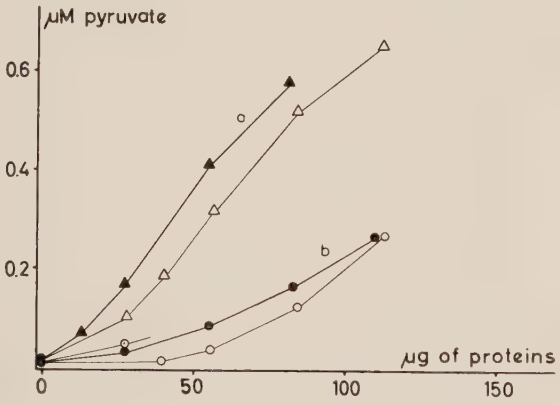

Fig. 4

Fig. 3.—Pyruvate kinase from soluble liver fraction, pH-curve.

Fig. 4.—Activity of pyruvate kinase from soluble liver fraction at varied concentrations. Curves *a*, pH 6.6. Curves *b*, pH 7.6; (○, △) 6 μM of ADP, (●, ▲) 3 μM of ADP, (⊙) 1.5 μM of ADP. It is noticed that the initial depression of the concentration curves was reduced at the lower ADP levels.

pared from rabbit muscle by the method of Szent-Györgyi [31] in the modification of Bowen and Kerwin [3].

(2) By a modification of the method for ATP determination by Pullman and Racher [27]. In this case the samples before incubation were further supplied with, per ml, 35 μM of glucose, and adequate amount of yeast hexokinase (prepared according to Berger *et al.* [2], with minor modifications), 0.3 μM of triphosphopyridine nucleotide (Sigma Co.) and 50 μg of glucose-6-phosphate dehydrogenase (Sigma Co.). As before, the reaction was ended, after 4 minutes of incubation, by the addition of EDTA. The formation of reduced triphosphopyridine nucleotide was measured at 340 mμ.

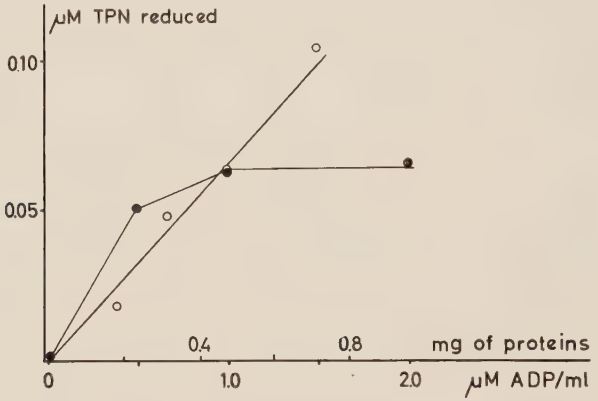

Fig. 5.—Activity of adenylate kinase from soluble liver fraction at varied concentrations of the extract (○), and of ADP (•). The analyses were performed by the hexokinase method.

The two analytical methods gave essentially the same results. Substrate and enzyme concentration curves, characterizing the system for adenylate kinase determination, are illustrated by Fig. 5.

RESULTS AND DISCUSSION

It was observed in previous experiments [17] that liver microsomes incorporated labeled amino acids into protein more efficiently when they were combined with supernatant fractions from regenerating livers than in combination with normal supernatant. This difference in incorporation capacity was observed both with normal microsomes and with microsomes from regenerating liver.

The concentration of the soluble fractions, which were used in these earlier experiments, were standardized on the basis of equal liver weights, and the proportions between microsomes and supernatant corresponded approximately to the conditions prevailing in the living cells. It was the aim of the present investigation to measure the effects of regeneration on the activity of the soluble liver components under more varied experimental conditions.

In the experiments reported here the activities of the soluble liver fractions were compared on the basis of equal protein contents. Since most of the incorporation experiments were carried out immediately after the preparation of the liver fractions, it was not possible, however, to determine the protein contents of these fractions before the start of the experiments. The supernatants, therefore, were added to the incubation samples at varied dilutions. Afterwards the incorporation values were compared diagrammatically for normal and regenerating liver supernatants which had been run in parallel in the same experiment (Fig. 1). The comparisons were always based on those parts of the concentration curves, where the samples still had contained acceptor microsomes in adequate excess.

The experiments showed that the capacity of the whole soluble fractions to promote the amino acid incorporation by liver microsomes increased during regeneration after an initial lag period of about 12–14 hours. The culmination occurred on the second day after the hepatectomy. The activity per mg of soluble protein was then about 60–80 per cent higher than in the normal liver.

The overall efficiency of the whole liver supernatants in incorporation systems of this kind may depend on a number of integrated factors. As was mentioned before, the ATP-generating system in these experiments was supplied with phosphoenolpyruvate as the source of high-energy phosphate. In order to transfer continually the phosphate from this compound to the adenosine monophosphate, which may ultimately be released by the activation reaction [13], the enzymes pyruvate kinase and adenylate kinase are required. Other components of the supernatant fraction, which obviously may influence the overall efficiency in the incorporation experiments, are the amino acid activating enzymes. It has been shown that these enzymes, in a hitherto unknown way are functionally associated with soluble RNA [14, 25]. Finally, GTP in some way is concerned with the incorporation process [19, 34].

The higher activity of the soluble fractions from regenerating livers was not due to increased endogenous concentrations of phosphoenolpyruvate or ATP, since these compounds were added separately to all incubation samples in adequate amounts.

The incorporation level increased, when GTP was added to the system (cf. [29]). However, the difference in activity between the soluble fractions from normal and regenerating livers was not influenced by this addition. In order to avoid possible interferences by endogenous guanosine phosphates, 0.2 μM of GTP were constantly included in the incubation samples in later experiments.

It seemed possible that the phosphate transferring enzymes involved in the ATP-generation might be responsible for the increased activity in regeneration by influencing the total efficiency of the soluble system. The activities of pyruvate kinase and adenylate kinase were therefore determined in soluble liver fractions from normal and regenerating livers. As is shown by Table II the soluble fractions did not show any increase in the activities of these enzymes during regeneration. However, the incorporation level increased considerably,

TABLE II. *Activities of pyruvate kinase and adenylate kinase in soluble fractions from normal and regenerating livers.*

The pyruvate kinase activities are expressed as μM of pyruvate released per mg of protein in 12 minutes at 35°C. The adenylate kinase activities (here determined by the hexokinase method) are expressed as μM of triphosphopyridine nucleotide reduced per mg of protein in 4 minutes at 25°C.

	Normal liver	Hours after hepatectomy				
		13	15	24	30	40
Pyruvate kinase, pH 6.6	8.1	8.0	8.2	7.5	5.8	7.0
pH 7.6	2.0	—	—	1.6	1.4	2.0
Adenylate kinase, pH 7.6	0.14	—	—	—	0.14	0.15

particularly in the samples with low concentrations of supernatant, when preparations of pyruvate kinase and adenylate kinase were included in the incorporation samples (Table I). The difference in activity between supernatants from normal and regenerating livers was not noticeably influenced by the addition of these enzymes, but in all subsequent experiments they were added to the incubation medium as a precaution. It was concluded that the difference in activity was not essentially due to dissimilarities in the rates of ATP-generation.

A number of experiments of this kind, performed at different periods after hepatectomy, are summarized in Fig. 6. The relative activities of the fortified soluble fractions used in these experiments have been calculated from diagrams of the type illustrated by Fig. 1, the activity of the normal soluble fraction being used as a reference. Some of the experiments collected in Fig. 6 have been performed with microsomes prepared from regenerating livers (30–48 hours), most of them, however, with microsomes from normal livers. In agreement with previous experiments [9, 17] the microsomes from regenerating livers constantly gave about twice as high incorporation values as the normal microsomes, but the relations between supernatant fractions from

normal and regenerating livers were not significantly influenced by the choice of test microsomes.

As a consequence of these experiments it seemed very likely that the increased activity of the soluble fractions after hepatectomy was related in some way to the functions of the amino acid activating enzymes. The study of these enzymes has recently been made possible by the application of two different methods [8, 10, 13]:

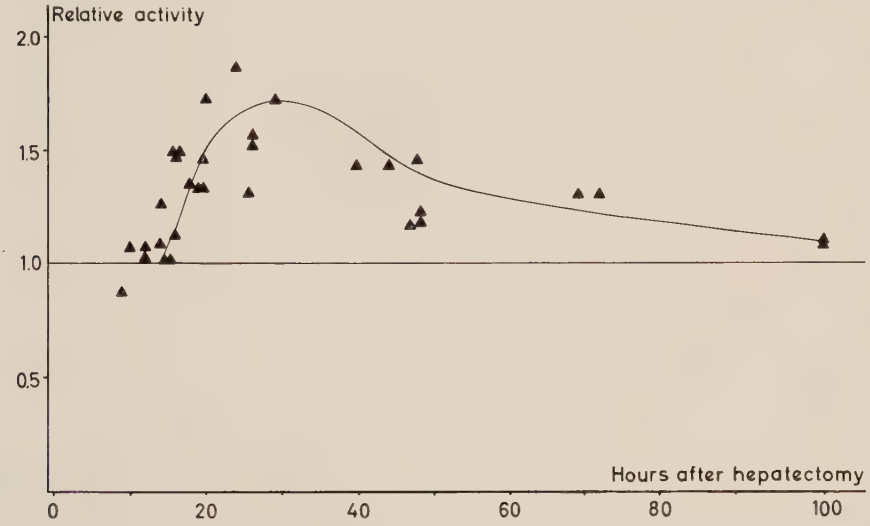

Fig. 6.—Activity of soluble liver fractions in incorporation systems, determined at different periods after partial hepatectomy by the method illustrated by Fig. 1. The soluble fractions from regenerating livers are compared with normal soluble fractions on the basis of equal protein content. The incorporation values obtained with normal soluble fractions in each experiment are represented by the horizontal line.

(1) By measuring the rate of isotope equilibration between ^{32}P-labeled pyrophosphate and ATP in the presence of an amino acid or a mixture of amino acids;

(2) By measuring the formation of hydroxamate, when ATP and an amino acid (or an amino acid mixture) is incubated in the presence of high concentrations of hydroxylamine.

These analytical methods have mainly been used in attempts to characterize the activating enzymes and to elucidate the mechanism of their action. As far as we are aware they have not been applied to comparative determinations of amino acid activating enzymes in crude tissue extracts.

As is well known [20] the soluble fraction of rat liver cytoplasm contains

considerable amounts of enzymes with the capacity for activating substrates other than amino acids, releasing pyrophosphate from ATP. Since substrates for these enzymes are available in crude supernatant fractions, a non-specific exchange of pyrophosphate (or formation of hydroxamate) takes place in such preparations even in the absence of added amino acids. These non-specific effects have usually been escaped by isoelectric precipitation of the

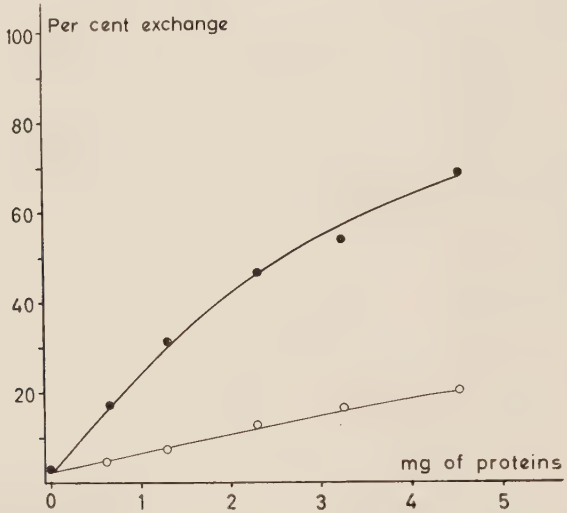

Fig. 7.—^{32}P-exchange between ^{32}P-pyrophosphate and ATP in the presence of varied amounts of dialysed normal soluble fraction. The incubation samples (1.2 ml) contained 4 μM of ^{32}P-pyrophosphate, 6 μM of ATP, 6 μM of $MgCl_2$, 60 μM of KF and 50 μM of tris buffer, pH 7.8. In the samples of the upper curve 3 μM of each of 12 amino acids had been included.

amino acid activating enzymes at pH 4.5–5.3 [8, 13, 30] or by treatment of the tissue preparations with ion-exchanging resins [8, 13]. The first of these methods is often particularly valuable, since it may be used for concentrating the enzymes.

In the present experiments both isoelectric precipitation with acetic acid and percolation through columns of the anion exchanger Dowex 2 were tried with good results in order to attain a satisfactory substrate specificity of the activation reaction. The soluble fractions from regenerating livers contained, however, increased amounts of lipids [32], and the deposition of this lipid material seemed to interfere with the quantitative yields, particularly in the isoelectric precipitations. Since the activities of the activating enzymes decreased rather slowly at 0°C, the supernatants, in most experiments, were instead freed from endogenous substrates by dialysis. Only

experiments of this kind have been included in this report. The dialyses were performed in 10 mm tubing (Visking) by continuous stirring for 17 hours against changed solutions of 0.1 *M* KCl and 0.007 *M* tris (0°C). The enzymatic activities were measured immediately after the dialysis by the procedure described previously. The substrate specificity of the exchange reaction, as measured by this method, is illustrated by Fig. 7.

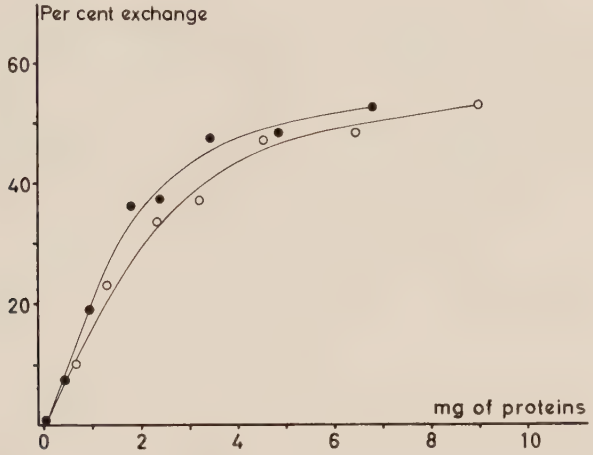

Fig. 8.—Amino acid dependent ^{32}P-exchange between ^{32}P-pyrophosphate and ATP in the presence of varied amounts of (○) normal, and (●) regenerating (49 hours) soluble fractions. System as in Fig. 7.

Soluble fractions from normal livers and from livers at different stages of regeneration were prepared and dialyzed in parallel, and the contents of activating enzymes were measured and compared on the basis of equal protein concentrations (Fig. 8). The relative activity values obtained from a number of experiments of this kind are summarized in Fig. 9. These values, like the values presented in Fig. 6, show a considerable scattering, which may largely be due to the well-known variability of the regeneration process. The enzymes, however, were constantly more active in the supernatants from the regenerating livers. This was true both when the amino acid mixture and when L-leucine alone was used as substrate in the enzyme analyses. Results in the same direction were obtained by experiments on hydroxamate formation. Only a limited number of such experiments were performed, however, and in most of these experiments the enzymes had been purified by isoelectric precipitation. The enzyme concentrations in dialyzed supernatants were suboptimal for the reaction.

A comparison between Figs. 6 and 9 suggests that the increased activity

during regeneration was less apparent, when the measurements were based on exchange experiments than when they were based on incorporation experiments. The slight divergency between the two series of results may be significant, since it was observed again in later experiments, in which dialyzed supernatants were used in parallel both for exchange and incorporation measurements (Table III).

TABLE III. *Activity for amino acid activation of dialysed soluble fractions from regenerating livers.*

Measured in parallel (1) by pyrophosphate-ATP exchange, (2) by ^{14}C-L-leucine incorporation in the presence of microsomes. The activity values of the soluble fractions from the regenerating livers are expressed in per cent of the values obtained simultaneously from normal control livers (cf. Figs. 1 and 8).

Hours after hepatectomy	Method for activity determinations: Incorporation of ^{14}C-L-leucine	Exchange of ^{32}P-pyrophosphate
24	159	125
48	116	105

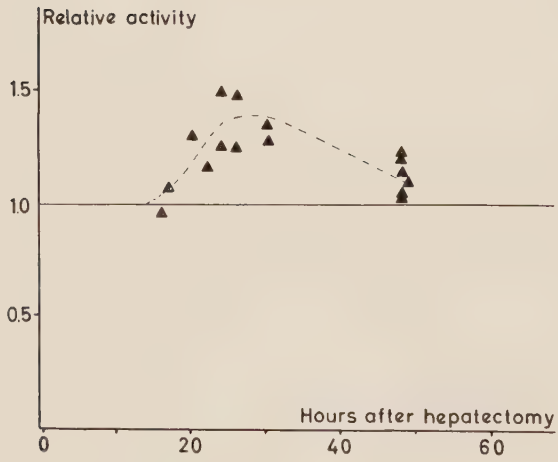

Fig. 9

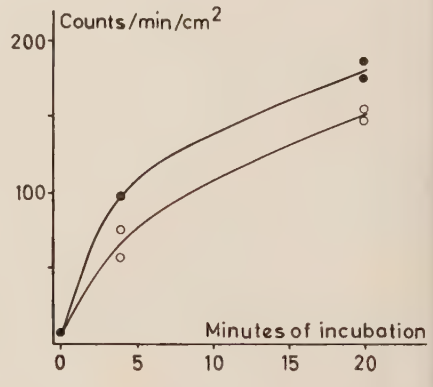

Fig. 10

Fig. 9.—The relative activity of soluble liver fractions for amino acid dependent pyrophosphate exchange at different periods after partial hepatectomy. The activities of the normal soluble fractions are represented by the horizontal line. The individual experiments were performed as is shown by Figs. 7 and 8.

Fig. 10.—Incorporation of ^{14}C-L-leucine into protein by normal microsomes in the presence of (○) normal, and (●) regenerating (48 hours) soluble fraction with a protein content of 9 mg.

It has been reported by Sachs and Waelsch [28] that liver supernatant contains a factor, which increases the stability of incubated microsomes. Since the incubation period was 15 minutes in most of the present experiments, it seemed possible that the discrepancy between the exchange and incorporation measurements might be due to a slightly increased functional stability of the acceptor microsomes in the samples with supernatant from regenerating liver. Preliminary experiments were performed to test this possibility by comparing the activities of the supernatants in the incorporation system at two rather different periods of incubation (Fig. 10). No significant difference was observed, however, between the short and long incubation periods.

TABLE IV. *Content of RNA (μg per mg of protein) in soluble liver fractions at different periods after partial hepatectomy.*

Normal liver	Hours after hepatectomy				
	9	12	15	25	44
14	14	13	13	16	16

It seemed possible that the discordance between the exchange and incorporation experiments might be related to the soluble RNA-fraction (S-RNA [14]), which is involved in the incorporation reaction, but not in the pyrophosphate exchange (cf. however [26]). The concentration of total RNA in whole soluble fractions from normal and regenerating livers was determined in a number of cases. Only slightly higher concentrations of RNA were observed in the supernatants from regenerating livers (Table IV). It is not known at present, whether the functional activity of the S-RNA fraction is correlated to the concentration of total RNA in soluble liver fractions. The activity of S-RNA is currently the subject of a more detailed study.

SUMMARY

Soluble fractions were prepared in parallel from normal and regenerating rat livers, and their capacity to activate amino acids for incorporation into proteins by liver microsomes was determined in incorporation systems using ^{14}C-L-leucine. When the relative activities of the supernatant fractions were compared at different periods after partial hepatectomy, a rise was observed after a lag period of about 12–14 hours. The rise was apparently about simultaneous with the rapid increase in microsomal activity, which occurs at the

beginning of the regeneration period. The increased activity of the soluble fraction, however, was quantitatively less pronounced than the activation of the microsomal material. The activity of the soluble fractions culminated on the second day after the hepatectomy. The rise was not primarily due to a higher efficiency of the ATP-generating system, but mainly to increased activities of the amino acid activating enzymes.

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METABOLISM OF GLUCOSAMINE IN THE EARLY SEA URCHIN DEVELOPMENT

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In the course of studies on the biochemistry of fertilization in sea urchins from this laboratory much attention has been paid to changes in carbohydrates, (cf. [23, 24, 9, 10, 11, 19, 20, 21, 22]). This writer has suggested a hypothesis according to which a certain depolarization of polysaccharides occurs on fertilization. The split products are supposed to react with unsubstituted amino groups of proteins.

Moreover Kavanau [14, 15] demonstrated that periods of breakdown of yolk proteins alternate with synthesis of embryonic proteins in the sea urchin development.

In view of these previous studies it seemed urgent to continue the analysis of the carbohydrates present in the egg. Moreover was it of interest to decide whether similar cyclic variations of carbohydrates occur as those demonstrated in the case of proteins and amino acids. The present paper refers to some results obtained from studies of glucosamine in the developing egg and larvae of *Paracentrotus lividus*. This was also the species which served as material in Kavanau's work. The results should therefore lend themselves to direct comparison.

MATERIAL AND METHODS

The egg and sperm of *Paracentrotus lividus* were prepared according to the customary procedure [10]. The jelly coat was removed completely by treatment with acidified sea water at pH 5.2 [26] and the egg suspension was washed several times by sedimentation in filtered sea water. The concentration of the eggs was determined by removing an aliquot of 0.5 ml from the suspension. This aliquot was diluted to 100 ml sea water and the eggs contained in 0.1 ml of the diluted suspension were counted. After fertilization in a concentrated suspension containing 70,000 eggs and 200,000 sperm cells per ml aliquots of 50 or 75 ml of the suspension of fertilized eggs were pipetted into each of 30 glass trays containing 400 to 500 ml of sea water. During the fertilization and the pipetting the egg suspension was maintained homogenous by an electrically driven multivaned glass stirring propeller. The culture trays were covered with glass plates and the embryos cultured at 20°C under slow rocking [16].

Each tray served for one chromatographic estimation and contained about five million embryos, the development stage of which was controlled and determined before fixation. The eggs and embryos of an entire tray were harvested quantitatively by centrifugation. The material was frozen by dipping the tube into a mixture of absolute ethanol and dry-ice and freeze-dried. The larvae were reared at the Station Biologique of Roscoff, France, in the summer 1954.

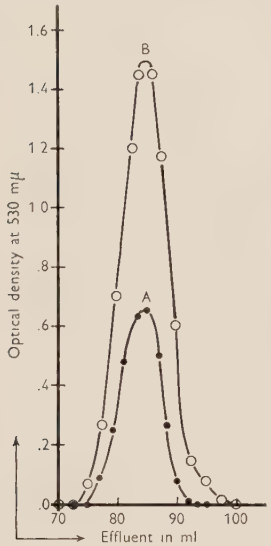

Fig. 1.—Separation and identification of glucosamine-HCl on Dowex 50 ion exchange resin. Column: 0.5 sq cm × 41 cm. Dowex 50: 200 to 400 mesh. Effluent: 0.3 *N* hydrochloric acid. Pressure: 140 mm of mercury. Curves: *A*, 500 μg glucosamine-HCl; *B*, 1640 μg glucosamine-HCl of hydrolysate of unfertilized *Arbacia lixula* egg without jelly coat.

The freeze-dried material was hydrolyzed with 6 *N* hydrochloric acid [2, 5] at 105°C for 3 hours. The hydrolysate was centrifuged, filtered through Jena glass filter 3G3 and the filtrate was placed in a small beaker and evaporated to dryness over soda-lime and sodium hydroxide in order to remove hydrochloric acid. The residue was then taken up in a small volume of distilled water and placed on a prepared column of Dowex 50 ion exchange resin [6] under extra pressure. The glucosamine was separated by 0.5 sq cm × 40 cm Dowex 50 column. Hydrochloric acid, 0.3 *M*, acting under constant pressure served as effluent. The column was connected to a fraction collector and the effluent collected in 3.5 to 5.0 ml fractions in the tube. The glucosamine was identified by means of two identical columns on which one known and one unknown specimen were tested in parallel runs, cf. Fig. 1. In order to determine which fractions contained reducing sugar, a little drop from each of the tubes was put on filter paper which was dried in a desiccator under reduced pressure over sodium hydroxide for about one hour, and the reducing sugars were detected by the technique of Trevelyan [25]. The spots containing glucosamine and other sugars appear black on the paper, as seen in Fig. 2. All tubes containing glucosamine solution (fractions 9-22) were collected in a 75 ml volumetric flask and the glucosamine was quantitatively determined according to Gardell's procedure, concerning details cf. [4, 6]. The hydrolysate of other monosaccharides (fractions 2 to 4) was collected in another flask and preserved in frozen state for further quantitative analysis of neutral sugars.

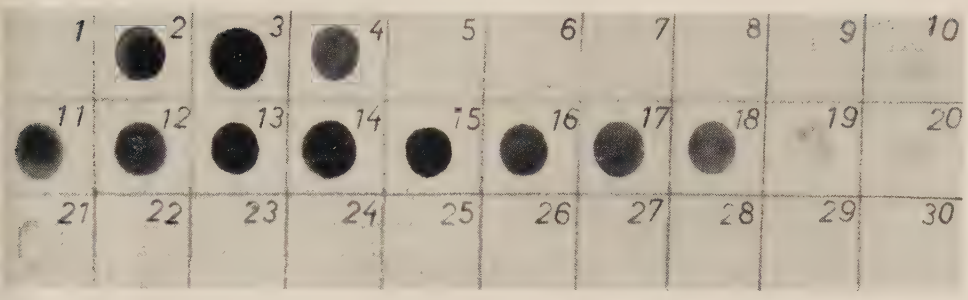

Fig. 2.—A series of tests to show which effluent fractions contain the reducing sugars. One drop of each effluent tube on the paper chromatogram. Volume of effluent in the tube approximately 5.0 ml. The numbers in the right corner of the square indicate effluent tubes. Fractions 2 to 4 contain neutral sugars (galactose, glucose, mannose etc.); fractions 10 to 21, glucosamine.

The amount of the glucosamine of each estimation was subjected to triple testing using the standard curve for glucosamine hydrochloride. The results were recalculated in μ-moles of glucosamine per embryo. The changes in glucosamine during the development until the first pluteus stage are represented in the curve of Fig. 3.

RESULTS

The average glucosamine content expressed as percentage of the dry weight of the unfertilized egg or as mg glucosamine per 100 mg nitrogen in the egg substance varies according to the sea urchin species studied. Thus the eggs of *Arbacia lixula* contain 0.77 per cent, *Echinus esculentus* 0.66 per cent and *Paracentrotus lividus* 0.84 per cent of glucosamine (Table I) per dry weight. For these determinations the *Arbacia* and *Paracentrotus* eggs were deprived of their jelly coats, whereas they were left intact in the *Echinus* egg. This may explain the lower glucosamine content which was found in *Echinus*.

The embryo does not seem to change its nitrogen content until the early pluteus stage. Nevertheless smaller variations occur (cf. [7], Fig. 22). The dry weight on the other hand may be also subject to variations for example by changes in content of inorganic ions. This may account for certain deviations from parallelism between the values for *Arbacia* eggs which are reported in columns 3 and 4 of Table I. In view of this the writer preferred in his main material presented in Fig. 3 to express the glucosamine values in μ-moles per egg or embryo. Kavanau [14, 15] followed the same principle when presenting his data concerning proteins and amino acids.

As an average of 8 estimations the unfertilized egg of *Paracentrotus lividus* was found to contain 61.8×10^{-5} μ-moles glucosamine. Half an hour after fertilization the content increased to 72.8×10^{-5} μ-moles per egg (average

of four estimations). Also in the prophase stage the glucosamine content (67.8 $\times 10^{-5}$ μ-moles) is higher than in the mature unfertilized egg but at the end of the first mitosis it assumes the same value as before fertilization. The glucosamine content then remains approximately constant until the 16-cell stage. There is apparently a minimum in the 8-cell stage, but the value is based here on only one estimation. It remains therefore doubtful if this deviation really is significant. In the stage of early blastulae, about 6 hours after fertilization a definite minimum obtains, representing a content of 48.6 $\times 10^{-5}$ μ-moles per embryo. Before hatching, however, almost the previous level is

TABLE I. *Content of glucosamine in the unfertilized sea urchin eggs.*

Hydrolysis by HCl of various N	Hydrolysis for time in hours	Glucosamine per cent of egg dry-weight	mg of glucosamine per 100 mg nitrogen
		Arbacia lixula[a]	
0.8	17	0.65	9.1
0.8	20	0.69	9.3
0.8	22	0.74	10.1
6	3	0.81	11.2
6	3	0.78	9.4
6	3	0.76	9.6
6	3	0.74	9.1
		Av. 0.77	9.7
		Echinus esculentus	
0.8	18.5	0.62	7.9
6	3	0.61	7.4
6	3	0.60	7.3
6	3	0.74	8.7
6	3	0.74	8.7
		Av. 0.66	8.0
		Paracentrotus lividus	
6	3	0.90	—
6	3	0.80	—
6	3	0.85	—
6	3	0.80	—
		Av. 0.84	—

[a] This material was kindly collected by Dr. A. Minganti.

attained (58.8×10^{-5} μ-moles). A new definite minimum is found between the early and late mesenchyme blastula stage (43.4×10^{-5} μ-moles). During the period from commencing to half invagination of the archenteron a maximum prevails with 56.4×10^{-5} μ-moles glucosamine per embryo. In the gastrulae with the archenteron fully invaginated a third minimum obtains which represents the lowest level of glucosamine during the early development (36.9×10^{-5} μ-moles). Beyond the late gastrula the glucosamine content increases to 50.0×10^{-5} μ-moles per larva.This value remains rather constant until the attainment of the pluteus stage.

Throughout the maxima and minima evolving during the development there is a general trend towards a decrease with respect to glucosamine content of

TABLE II. *Developmental stages prevailing at different times after fertilization of* Paracentrotus lividus *eggs at 19°C.*

Developmental stages	Average time in hours and minutes	Range of variation of the development time in hours and minutes
1. Unfertilized egg	0.00	0.00
2. Syncarion stage	0.30	0.25– 0.35
3. Prophase stage	1.00	0.50– 1.05
4. Dumb-bell stage	1.20	1.15– 1.25
5. 2-cell stage	1.30	1.25– 1.50
6. 4-cell stage	2.20	2.00– 2.50
7. 8-cell stage	3.10	2.50– 3.30
8. 16-cell stage	4.00	3.50– 4.05
9. 32-cell stage	4.40	4.30– 4.50
10. 64-cell stage	5.10	4.50– 5.20
11. Early blastula	6.20	6.00– 6.55
12. Blastula	9.30	9.00–10.00
13. Late blastula	10.30	10.20–11.00
14. Hatching	12.00	11.50–12.05
15. Early mesenchyme blast.	13.30	12.00–15.30
16. Late mesenchyme blast.	15.00	14.00–16.00
17. Flat. to invaginating	17.00	15.00–19.30
18. $\frac{1}{2}$ invaginate	20.35	18.30–23.00
19. $\frac{3}{4}$ invaginated	22.40	19.20–25.00
20. Late gastrula	24.30	21.15–26.45
21. Early prism	27.00	25.00–31.00
22. Late prism	29.00	27.00–32.20
23. Early pluteus	33.00	32.20–33.30
24. Pluteus	40.00	33.00–43.00

the egg or embryo. It is about 24 per cent lower in the pluteus larva as compared with the unfertilized egg, whereas if the pluteus larva is compared with the fertilized egg in stage 2 (syncarion) the decrease in level amounts to 46 per cent, as it is illustrated in Fig. 3.

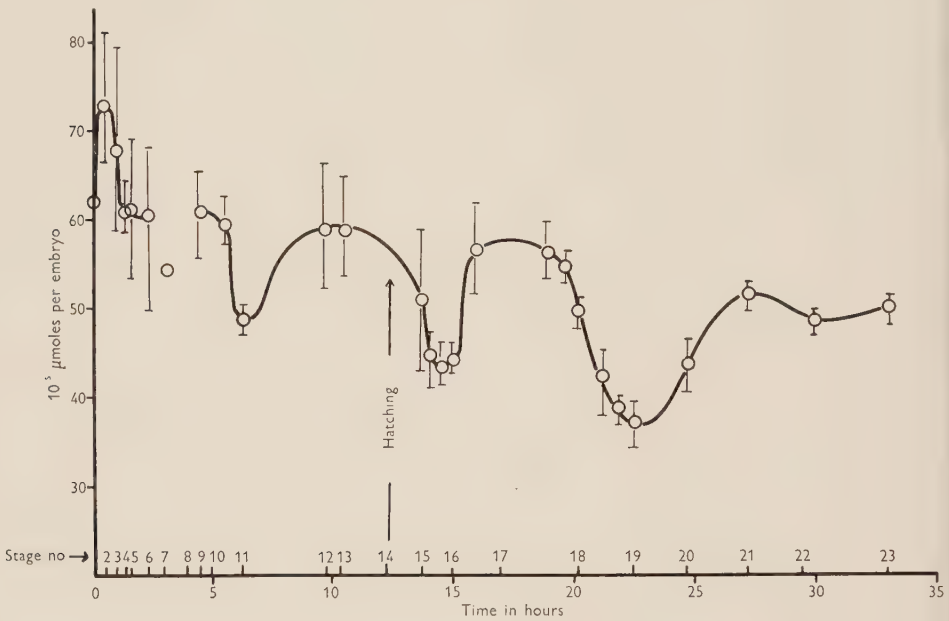

Fig. 3.—Change of glucosamine content in μ-moles per embryo during the development of the sea urchin *Paracentrotus lividus* larva. Every circle of curve presented an average value of glucosamine from four to eight estimations obtained from series A, B, C and D. Each series consists of 30 trays fertilized simultaneously and reared under identical conditions. The vertical lines indicate the range of the variation of the values obtained at different stages of development. For detailed indications of the development stages corresponding to the time values, cf. Table II.

Two estimations made on *Arbacia* eggs one hour after fertilization gave the values 0.94 and 0.84 per cent glucosamine of the dry weight. On comparison with the values found in Table I it seems probable that an increase in glucosamine content occurs after fertilization also in the *Arbacia* egg.

DISCUSSION

A comparison with the data presented in Fig. 3 and Kavanau's data referring to proteins and amino acids ([15], Fig. 1) indicates a clear correlation between these two groups of data. A decrease in the glucosamine content coincides

with an increase in protein content, i.e. synthesis of embryonic protein. There is only one reservation to be made, viz. with respect to the stages before cleavage where Kavanau's data are very scarce. As a consequence no comparison is as yet possible in these stages. Otherwise the decrease of glucosamine content in the early blastula stage (Fig. 3) corresponds very well to the increase in total protein demonstrated for this stage by Kavanau [15].

The same applies to the decrease in glucosamine content found in the early mesenchyme blastula stage. Likewise the decrease in glusosamine content around the stage of half invagination goes along with an increase in total protein in Kavanau's experiments.

If this correlation is regarded as established how is it to be explained?

The present research is certainly not concerned with free glucosamine but with a split product of mucopolysaccharides. The gradual decrease of glucosamine during development may indicate that it belongs to the yolk material which is gradually consumed, cf. Kavanau [15] for the changes of yolk proteins.

In view of the correlation discussed it may be tentatively suggested that glucose-containing polysaccharides serve as donors or acceptors of aminogroups. In this context it may be recalled that Örström [27, 28, 29] demonstrated an increased production of ammonia upon fertilization of the *Paracentrotus* egg. Hutchens *et al.* [8] showed that a production of ammonia may also occur in the course of the following development. Recently Örström's results have been confirmed by Ishihara [12]. Like Örström he considers that the ammonia arises by deamination of adenylic acid. The production of ammonia during this stage would thus depend on the presence of an adenosine deaminase, an enzyme the quantity of which has been shown to remain constant during the different stages of development [7]. It must be left open as to whether the ammonia production demonstrated by Örström and Ishihara is correlated to the increase in glucosamine content observed after fertilization.

Another group of enzymes which may be concerned in deamination and amination processes are the cathepsins. Fulton [3] showed that these enzymes are able to split certain amides when tested at pH near 5.0. On the other hand Jones *et al.* [13] demonstrated that at pH values around 7.5 the predominant reaction is not a hydrolysis but rather a transamidation leading to the formation of a polypeptide amide. A polymerization occurs through successive transamidations, in each of which an α-amide is converted into a peptide and ammonia, cf. [3]. Fulton and coworkers have demonstrated that also other proteolytic enzymes, like papain and trypsin, are able to bring about trans-

amidations. According to Fulton these phenomena may have some significance for the problem of protein synthesis. In this context it is of interest that Lundblad [17, 18] has demonstrated that an activation of proteolytic enzymes of the type cathepsin II occurs upon fertilization.

If glucosamine serves as an amine-donor during periods of protein synthesis, glucose residues of mucopolysaccharides may, on the other hand, act as amine acceptors. It should be pointed out that the substance which is determined as glucosamine in the present work may in reality—before hydrolysis –be a combination of glucose residues and amino groups by which 1-amino-glucose, Schiff's bases, etc. are formed. This would be in agreement with the writer's scheme referred to above ([11] Fig. 13), according to which a splitting of polysaccharides occurs upon fertilization with ensuing binding of the amino groups in proteins to the 1-position in glucose residues of mucopolysaccharides. Recently Boström and Perlmann [1] made the significant discovery that the sea urchin egg has a rather high content of sialic acid. One must thus expect that a certain part of the glucosamine content of the sea urchin egg or embryo may exist as a component of sialic acid. The glucosamine estimated in this research represents probably fragments from several complex substances present in the living cell.

SUMMARY

By aid of chromatographic assay the glucosamine content has been determined in different stages of development of the sea urchin *Paracentrotus lividus*. Some data are also given for eggs of *Arbacia lixula* and *Echinus esculentus*.

There is a general trend towards a decrease of the glucosamine content per egg or embryo during the development. It is about 24 per cent lower in the pluteus larva as compared with the unfertilized egg and 46 per cent lower in the pluteus stage if it is compared with the fertilized egg in the syncarion stage, cf. Fig. 3. The decrease is, however, not a steady one but the values fluctuate showing certain pronounced maxima and minima, as illustrated in Fig. 3. The fluctuations compare very well with those demonstrated by Kavanau for total protein in *Paracentrotus* embryos. A decrease in the glucosamine content coincides with an increase in total embryonic protein and an increase with a breakdown of yolk proteins. It is therefore tentatively suggested that glucosamine plays a role in protein synthesis. It might act as an amine donor or acceptor. Reference is made to the work of Fulton and co-workers which points to the role of peptide amides in protein synthesis.

The glucosamine estimated in this study forms probably part of mucopolysaccharide molecules. The substance determined as glucosamine may—before hydrolysis—partly represent a combination of glucose residues and unsubstituted amino groups. Its possible role as a component of sialic acid is emphasized.

The writer wishes to express his most cordial thanks to Professor John Runnström for his kind criticism, valuable advice and correction of the manuscript. Thanks are also due to Professor P. Drach, Station Biologique, Roscoff, for his never-failing generosity.

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BRIEF NOTES

THE CHROMATOID BODY OF *ENTAMOEBA INVADENS*

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Chromatoid bodies are found in the cysts of nearly all *Entamoeba*. They are rod or bar shaped cellular inclusions, up to 10 μ long, which stain deeply with basic stains. Deutsch and Zaman, using the electron microscope, found that in *Entamoeba invadens* they consist of small particles (about 200 Å in diameter) arranged in a crystalline pattern. There is some indication that these particles are composed of smaller units. Chromatoid bodies have also been observed in the trophozoite of *Entamoeba invadens*, where they are smaller and more numerous than in the cyst [1] and in *Entamoeba histolytica* [2]. Ray and Sen Gupta [8] report that in *Entamoeba histolytica* the bodies contain varying amounts of DNA and RNA. The bodies have been regarded as excess chromatin thrown off during division [4], or as food stores [2, 5]. According to Ray and Sen Gupta [8] they play a part in the deposition of the cyst wall. We have attempted a more comprehensive histochemical study of these interesting inclusions and an analysis of their changes in relation to the life cycle of the organism

Material and Methods.—The organisms were cultured in Jones' medium at 25°C [6]. Fixation was mainly carried out in Schaudinn's fluid, but also Carnoy's 1 and 2, Champy's, Flemming's, Zenker's, Serra's fixatives, osmium tetroxide, formalin and sublimate acetic acid were used [3, 9]. Table I contains a list of the histochemical tests carried out.

Trophozoites treated with ribonuclease in the living state (see Table I, B, col. 5) were studied also in the electron microscope. They were first mixed in osmium tetroxide, dehydrated, embedded in a methacrylate mixture according to standard procedure and sectioned at 250 Å.

For investigation of the cyclic changes of the bodies in the trophozoite, samples were taken in intervals starting from the third day after inoculation. In each sample the number of bodies larger than 0.5 μ was counted and the length of the longest axis was determined. The cysts were studied in a similar way, but the whole culture was treated with distilled water for 24 hours to get rid of the trophozoites and to avoid the formation of new cysts.

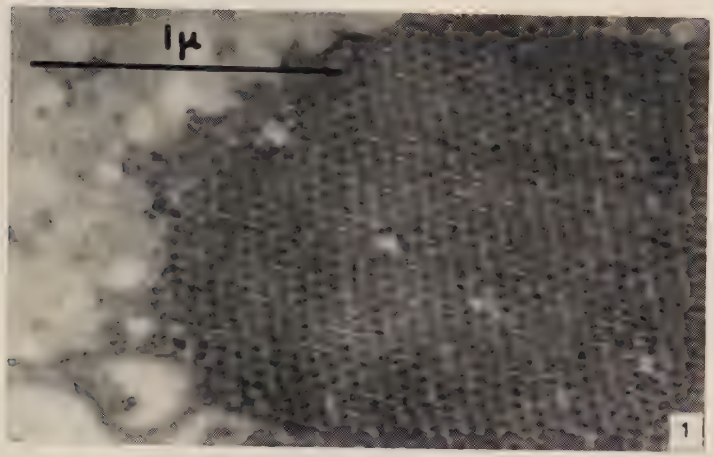
Results and Discussions.—The histochemical tests (see Table I) indicate that the bodies consist mainly of large amounts of ribonucleic acid and some unspecified proteins. We could not demonstrate the presence of desoxyribonucleic acid as in *Entamoeba histolytica*. Ribonuclease apparently did not affect the cyst (Table I, B, col. 5), as presumably the large enzyme molecules could not penetrate through the cyst wall. A preliminary electron microscope study shows that the globular particles which form the bodies, appear to be flattened after treatment with the enzyme (com-

TABLE I. *A. Staining reaction.*

Test	Reference	Reaction of chromatoid bodies	Casual observation
1. Basic stain.			
(a) Heidenhain's iron haematoxylin.	Dobell [10]	Deeply stained	Nucleus as a peripheral ring of chromatin with a central nucleolus.
2. Glycogen detection tests.			
(a) Bauer Feulgen.	Hale [12]	Negative	Glycogen mass in cysts and staining spots in trophozoites.
(b) Best's carmine.	Bensley [2]	Negative	
(c) Periodic acid Schiff's.	Hotchkiss [15] McManus [18]	Stained membrane prevents definite statement but probably negative	Deeply stained membrane prevents visualisation of contents.
3. Protein tests.			
(a) Berg's ninhydrin.	Glick [11]	Doubtfully positive	Rest of the cytoplasm lightly stained.
(b) Bromphenol blue.	Mazia *et al.* [19]	Deeply stained	Rest of the cytoplasm stained.
4. Lipid tests.			
(a) Sudan black.	Burdon [6]	Negative	Fat droplets in cytoplasm.
(b) Neutral red.	Ray and Sen Gupta [24]	Negative	Fat droplets in cytoplasm.
5. Nucleic acid tests.			
(a) Feulgen nuclear reaction.	Wenrick [25] Rafalko [23]	Negative Negative	Nucleus only very poorly stained.
(b) Toluidine blue.	Michaelis [20]	Deeply stained	Nucleus as in 1.
(c) Methyl green pyronin.	Kurnick [17]	Pyronin positive methyl green negative	Nucleus methyl green positive pyronin negative.
6. Fluorescent microscopy.			
(a) Acridine orange. (b) Trypaflavin.	Armstrong [1]	Bright orange	Nucleus green yellow.
(3,6-Diamino-10 methyl-acridinium chloride)	De Bruyn *et al.* [7]	Orange	Nucleus yellowish green.

TABLE I. *B. Extraction of nucleic acids followed by staining with toluidine blue or methyl green pyronin.*

Test	Reference	Reaction of body after extraction	Controls (H_2O = Distilled water)
1. Hot trichloracetic acid.	Pollister and Ris [22]	Unstained	Hot H_2O. Slight reduction in basophilia of bodies. Cold H_2O. Stained normally. Cold TCA. Stained normally.
2. 10 % perchloric acid at 4°C.	Di Stephano [9]	Unstained.	H_2O at 4°C. Bodies normally stained.
3. Hot normal hydrochloric acid.	Vendrely-Randaval [25]	Unstained	H_2O at 60°C. Slight reduction in basophilia. Cold H_2O and HCl. Bodies stained in the normal way.
4. Ribonuclease digestion on fixed material.	Brachet [3]	Unstained after 4 hours' treatment	Results varied with the fixative used. Schaudinn's practically ineffective, as was Zenker's fixed material. Carnoy's and Serra's both showed big reduction in basophilia.
5. Ribonuclease on living material treated for 4 hours in a solution of 10 mg ribonuclease in 10 cc water.	Brachet [4] Brachet [5]	Substantial loss in basophilia in trophozoite bodies but not in cyst. Changes in the microstructure (EM)	No loss of basophilia with H_2O.



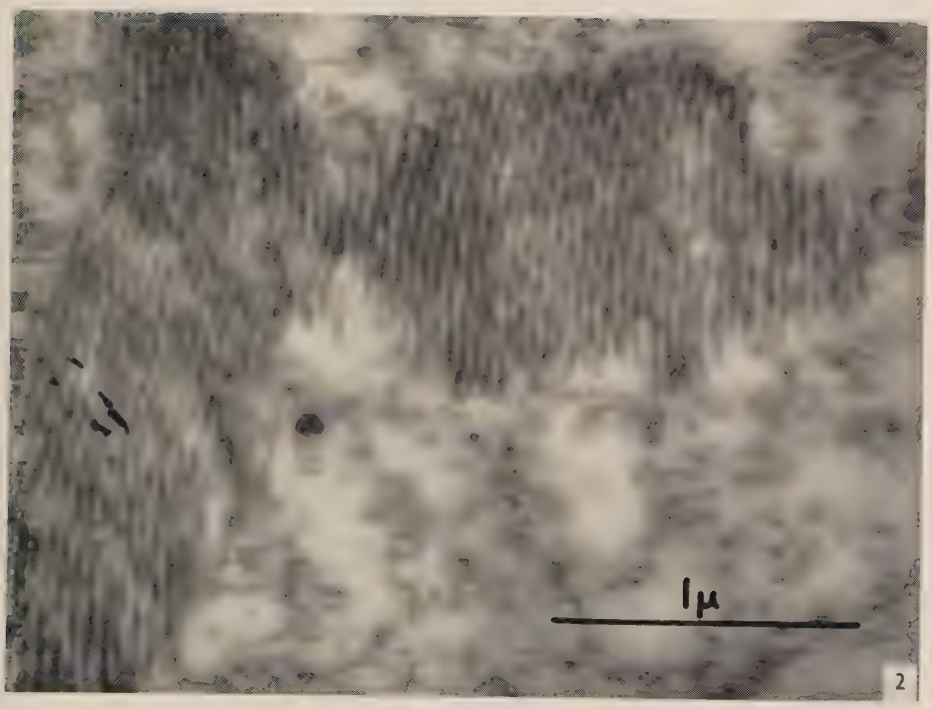


Fig. 1.—Chromatoid body. Fixation osmium.
Fig. 2.—Chromatoid body. Treated with ribonuclease. Fixation osmium.

pare Figs. 1 and 2). They consist now of linearly arranged subunits which are represented in the electron microscope as electron transparent dots (diameter about 70 Å) with electron transparent centres (diameter about 30 Å) (Fig. 3). It is interesting to note that in formalin fixed, but untreated cysts, the particles appear also to be flattened (Fig. 4).

TABLE II. *Average number and size of bodies (larger than 0.5 μ) in trophozoite and cyst.*

Trophozoite			Cyst		
Day	Number of bodies per cell	Average length μ	Hour	Number of bodies per cell	Average length μ
3	0.5	1.7	4	1.4	4.8
4	0.6	1.8	16	2.1	4.9
5	0.3	2.4	24	1.7	4.2
6	1.5	2.2	48	2.1	4.3
7	3.5	2.2	72	3.7	3.0
8	3.3	2.8	96	4.4	2.3
9	2.3	2.8	120	3.3	2.1
10	4.4	3.2			
11	2.9	4.3			
12	3.0	4.2			

Analysis of the changes of the bodies larger than 0.5 μ in relation to the life cycle shows an increase of their number and their size with increasing age of the culture (Table II). The point of maximum size is reached in the late trophozoite and the early cyst. The number, however, drops during encystment. With increasing age of the cyst, the number increases again and the size decreases. Electronmicrographs show that the early trophozoite contains a great number of bodies smaller than 0.5 μ, and it appears likely that the larger bodies are formed by aggregation of the smaller ones, and that the total number of bodies actually decreases in the trophozoite until encystment, as no small bodies were detected in the early cyst [1]. The size and the chemical composition of the particles indicates that they could have a function similar to the RNA particles in other cells [7], but there is also a certain resemblance between the particles and viruses, and we shall try to clarify by further investigations the significance of these peculiar cellular inclusions.

Summary.—Histochemical studies have shown that the chromatoid bodies consist mainly of ribonucleic acid and some unspecified proteins.

A preliminary electron-microscope study has shown that the globular particles which form the bodies are flattened after treatment with ribonuclease.

The average size of the bodies over 0.5 μ increases with the age of the culture in the Trophozoite and decreases again in the cyst.

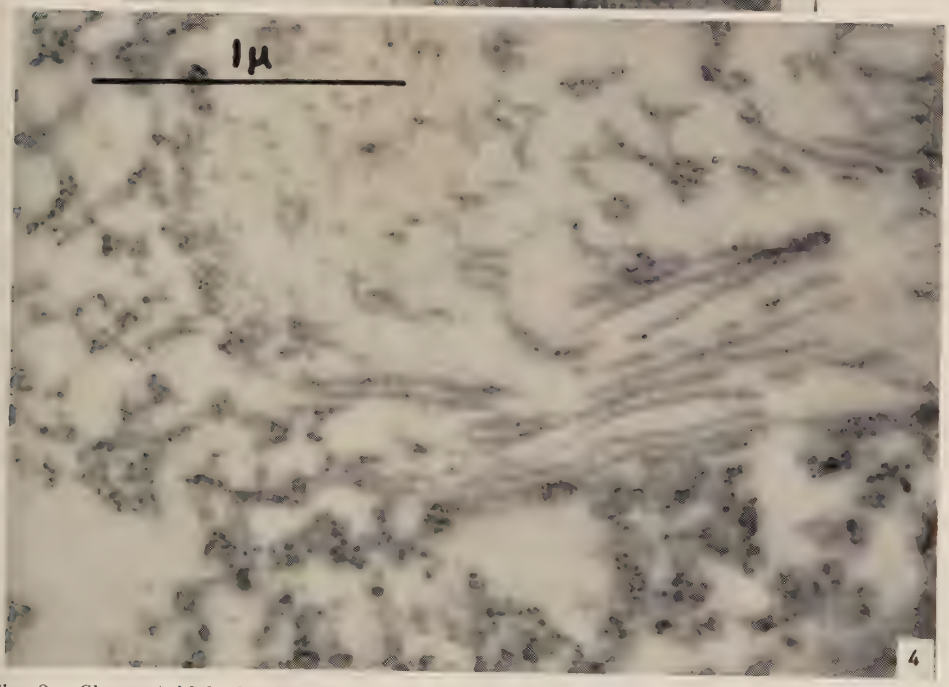


Fig. 3.—Chromatoid body. Treated with ribonuclease showing fine structure of the particles. Fixation osmium.

Fig. 4.—Chromatoid body. Fixation formalin.

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THE MOTION OF PLANT SPHEROSOMES

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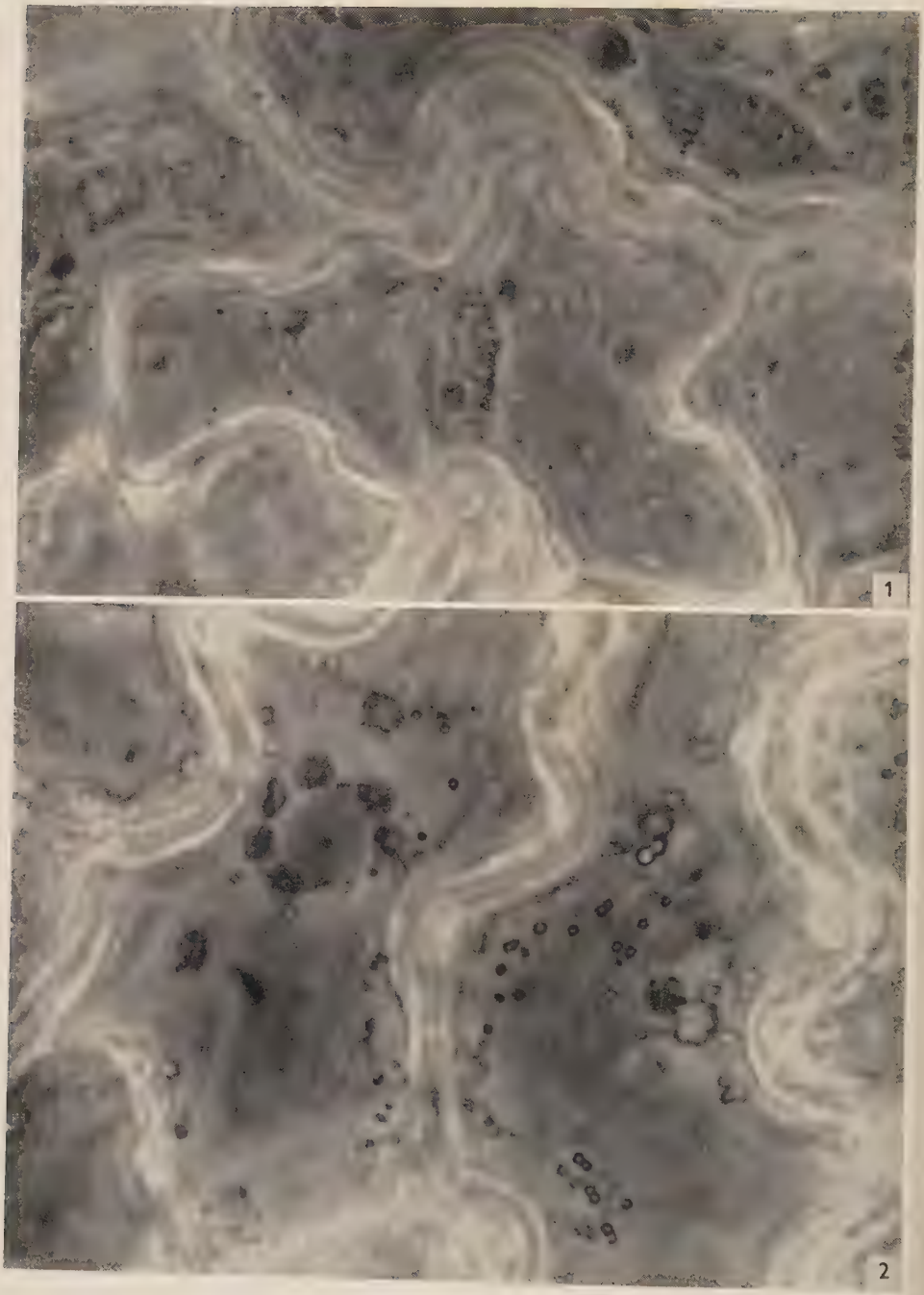
The motion of spherosomes may ideally be studied in epidermal cells from the lower side of rosette leaves in *Campanula persicifolia* L., because there they are large in size and few in number. In the studies of temperature effects on living cells Alexandrov [1] had found in this material the motion of spherosomes to be a sensitive criterion of cell vitality.

When the epidermis is stripped off, mounted on a slide with the cutinized side down in a solution of sucrose 0.26 *M* and examined with a phase contrast microscope, the spherosomes are seen as opaque white globules 0.7 μ in diameter (Fig. 1), or as dark-rimmed spheres (Fig. 2). These globules and spheres are distinct from granular and rod-shaped mitochondria which are found in a different layer of cytoplasm and identified by reversible coloring and decolorization after treatment with Janus green B [2]. The motion of spherosomes is sluggish at first (in all probability due to trauma), but it soon increases to normal. Not all of the spherosomes move at the same rate. An average speed is about 3 μ per second. Certain forms move slower and intermittently, others move faster and by-pass the slower ones. Although in some parts of the cell the motion appears to be haphazard, little balls moving in different directions, there is, nevertheless, an overall course of motion. This is witnessed by the streams and paths of particles visible in the parietal layer of the cytoplasm and across the width or length of the cell. The spherosomes also enter the cytoplasmic trabeculae which cross the central vacuole towards the nucleus, where they may become stationary for a period of time, or glide along its surface. Still other spherosomes move alongside plastids, which are often arranged in a circle about the nucleus (Fig. 2). In all of these locations individual spherosomes are continually associating on separating from one another. A particle comes in contact with another either through a rapid striking motion or a slow approach, and for a time the two form a diplosome (lower foreground, Fig. 2). Such associated spherosomes may separate at once, or the diplosome may move a short distance and then turn about 90—or more often 180—degrees after which separation would proceed abruptly. When the diplosomes break apart into two spherosomes without preliminary rotation, they usually continue to travel in the same direction, and may again become associated into a diplosome. However, the turning of the diplosome for 180 degrees usually results in rapid separation of the spherosomes which move away in different directions. Thus separated, the spherosomes may again strike others and become associated into groups of three (Fig. 1), or form short chains of four or more links. These short chains may continue to move in the same direction. The end links may be picked up by other spherosomes to form new diplosomes which travel in an opposite direction. Occasionally, one of the central spherosomes of a chain may become associated with a passing spherosome to break the chain in the middle. This may suggest that a free moving spherosome has a greater force of attraction, as compared with a similar spherosome situated in a short chain, described above, and subjected to attraction between the neighboring spherosomes of the chain.

Sutcliffe [3] had expressed the opinion that mitochondria may represent particles responsible for the transport of adsorbed ions from the inner membrane of the protoplast, across which secretion occurs, into the vacuole. In the material studied the mitochondria are located in a restricted layer of the cytoplasm and may not be sufficiently mobile to account for such transport. The occurrence of spherosomes in the parietal layer of the cytoplasm in close proximity to the peripheral, predominantly lipoid membrane, and their ability to reach through the cytoplasmic stream all other parts of the cell suggests they might be better suited than mitochondria for the transport of substances throughout the cell.

With the support of a grant from the American Philosophical Society.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A TECHNIQUE FOR THE STUDY OF MYOGENESIS *IN VITRO*

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Received September 6, 1958

The differentiation into muscle elements of cells originating in explants of embryonic muscle tissue has been repeatedly studied. The literature has recently been reviewed by Godman [2] and individual references will not be given here. In the case to be described differentiation was both constant and rapid and the method may be suitable for studies of specific aspects of the phenomenon of differentiation.

Differentiation was observed in monolayer cultures of chick embryo cells, grown in sealed plastic dishes [1] and overlain with nutrient agar. However, they could equally well be grown in glass petri dishes in an atmosphere of 5 per cent carbon-dioxide.

The monolayers were prepared by a method which has become standard laboratory practice. A cell suspension was prepared by maceration and trypsin digestion of chick embryos, aged 11 to 13 days, from which the eyes and claws had been removed. Digestion was carried out at 37°C using a solution of 0.25 per cent trypsin in phosphate-buffered saline. Each dish contained an initial inoculum of $5 \cdot 10^5$ to 10^6 cells in 4 ml of growth medium consisting of 4 parts horse serum, 5 parts Earle's saline, and 1 part calf-embryo extract with penicillin and streptomycin. After incubation for 48 hours at 37°C the cells had grown to produce a monolayer consisting largely of fibroblast-

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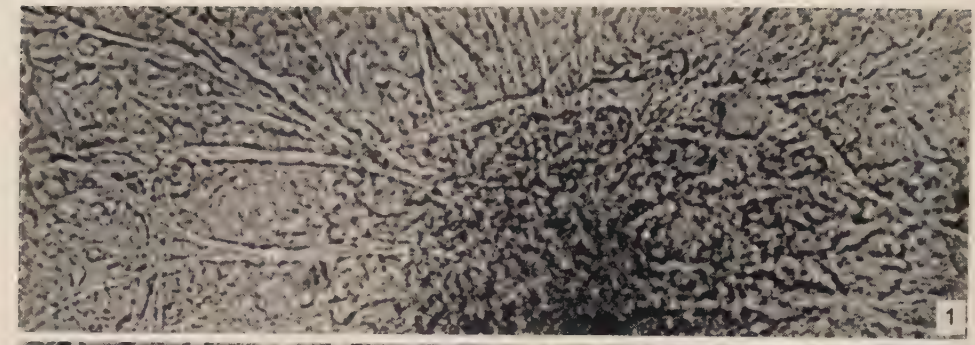
Figs. 1–2.—Photomicrographs of living cells taken when the motion had subsided. Dark phase contrast, neofluar objectives Ph 100/1.30, K ocular 8 (Fig. 1), and Ph 40/0.75, K ocular 20 (Fig. 2). ×1530.

Fig. 1.—Spherosomes appear as opaque white globules seen as single spherosomes, diplosomes, and triplosomes.

Fig. 2.—Spherosomes have the appearance of dark-rimmed bodies. In the cell to the right a path of spherosomes is visible in the parietal layer of the cytoplasm. Diplosomes, a triplosome, and single spherosomes are seen in the lower foreground. In the cell to the left chloroplasts surround the nucleus, and the spherosomes are recognized in the vicinity.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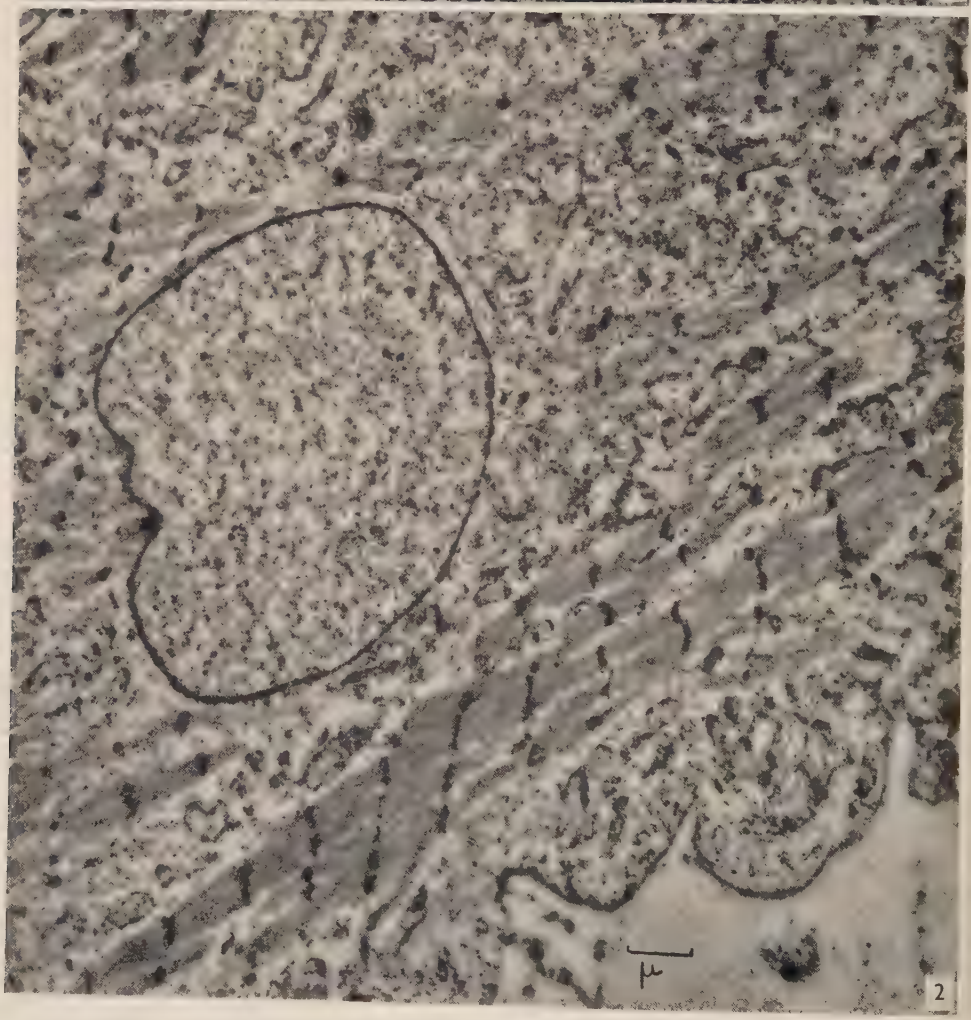
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like cells. At this time the medium was removed and replaced with an agar medium as follows: 4 parts horse serum, 2.5 parts double-strength Earle's saline, 1 part calf-embryo extract, and 2.5 parts of a solution of washed agar (3.6 per cent in double-distilled water). To this medium 3.0 per cent adult chick serum was added to make the final solution.

After three days of continued incubation at 37°C many fibres (sarcoblastic ribbons), some of them obviously striated, were present in the cultures and underwent contraction when observed in the light microscope. The fibres frequently radiated from foci of apparent giant cells (Fig. 1). The cultures remained healthy under these conditions for up to ten days without a change of medium. In the absence of the agar overlay this degree of differentiation was never observed during the same experimental period.

To investigate the nature of the fibres a study of the cultures was carried out with the electron microscope, using the following fixation method. After removal of the agar layer, the material was fixed *in situ* with osmic acid vapour for ten minutes; the cell layer was then picked up with a spatula and fixation completed in a solution of 2 per cent buffered osmic acid for one hour. The sheets of cells were passed rapidly through 70 and 90 per cent alcohol, dehydrated and embedded in methacrylate in the usual way. On sectioning, the blocks were oriented to obtain the maximum number of cells. In this way it was possible to observe some stages in myogenesis. Cells were found at an apparently early stage, in which diffuse masses of myofibrils filled the cytoplasm, without organization of bundles or of striations. Presumably later stages were observed, in which organized bundles of striated muscle were found (Fig. 2). This method could be applied to a more detailed study of muscle fibre formation.

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Fig. 1.—Monolayer culture under agar, showing muscle tissue. 200 ×.

Fig. 2.—Electron micrograph of a cell showing striated muscle taken from a culture undergoing differentiation. 8,000 ×.

SOMATIC ASSOCIATION OF THE POSITIVELY HETEROPYCNOTIC X-CHROMOSOMES IN FEMALE MICE *(MUS MUSCULUS)*[1]

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A SEX difference in the morphology of somatic interphase nuclei of mammals was first noted in 1949 by Barr and Bertram [1]. This observation has since been confirmed in many laboratories. The current conception of the so-called sex chromatin has been described in some detail by Moore and Barr [3] and by Klinger [2]. In various species of *Primates*, *Carnivora* and *Artiodactyla*, a distinct chromocenter of certain size is clearly visible in the somatic nuclei of females, whereas a corresponding body of comparable size is seldom visible in cells of the males of these species. Such evidence suggests that the so-called sex chromatin of female cells is formed by a fusion of heterochromatin portions of the two X-chromosomes, while the XY-chromosomes of the male do not form a structure of comparable size. Unfortunately, our present knowledge of these species' sex chromosomes is such that the above suggestion can neither be proved nor disproved at present.

Our previous studies on male germ cells of *Mus musculus* have revealed that both the X- and the Y-chromosomes of this species are totally heterochromatic and that they remain positively heteropycnotic during spermatogonial interphase [4]. During studies on female germ cells of *Mus musculus*, many mitotic figures of Graafian follicle cells were encountered. At prophase, the two X-chromosomes in positively heteropycnotic state were found, without exception, to be in end-to-end association. Since somatic association of the sex chromosomes in mammals does not appear to have been actually observed previously, our observations are recorded briefly below.

[1] Supported in part by both PHS Grant 2597 and Damon Runyon Grant 428.

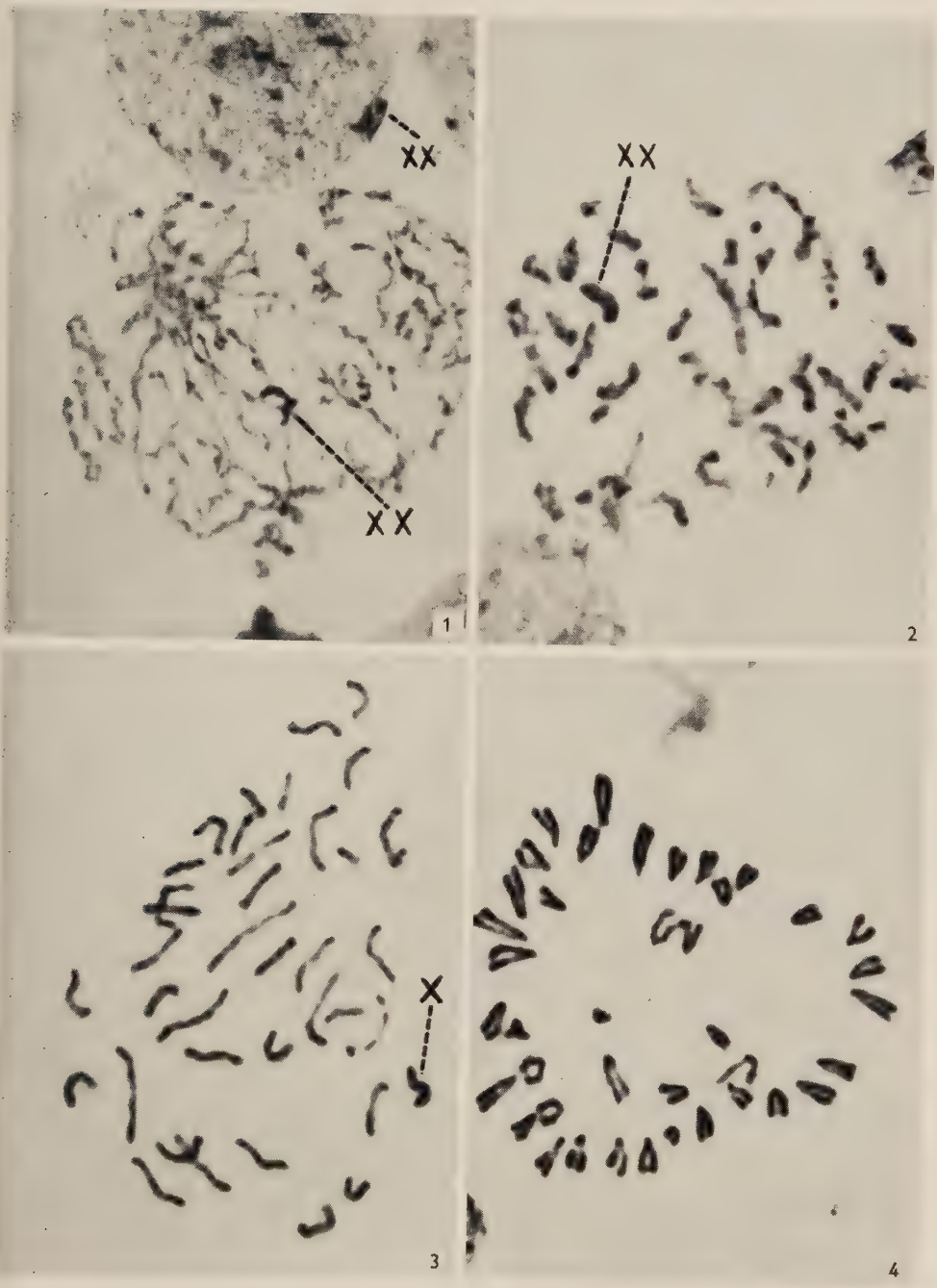
Figs. 1, 2, 3 and 4 are photomicrographs of Graafian follicle cells of mice, taken by Leitz Panphoto, 90 × 10.

Fig. 1.—The upper figure shows an interphase nucleus, demonstrating the so-called sex chromatin attached to the nuclear membrane. The appearance of the sex chromatin suggests that it consists of two chromosomes in a positively heteropycnotic state paired side-by-side. Also shown is an early prophase figure clearly revealing the end-to-end association of the two X-chromosomes in the positively heteropycnotic state.

Fig. 2.—A late prophase figure. The two X-chromosomes in the positively heteropycnotic state are still in end-to-end association.

Fig. 3. A prometaphase figure. One X-chromosome in a positively heteropycnotic state is no longer found with the other. The other X-chromosome and one autosome distributed high above the field are not included in this photomicrograph.

Fig. 4.—A metaphase figure. Since the autosomes are now condensed to the same degree as the X-chromosomes, the X-chromosomes are no longer readily recognizable.


XX
XX
1
XX
2
X
3
4

Six to twelve month old female mice of C_3H, AKN and Swiss strains were used. The squash preparations of ovarian tissues were prepared according to our previously described Feulgen squash technique [4].

A considerable percentage of follicular cells in interphase demonstrated the so-called prochromosome nucleus which contains approximately 39 chromocenters. A large outstandingly heteropycnotic chromocenter of bipartite nature stands out from the remaining 38 small chromocenters which apparently correspond to the centromeric heterochromatin of the 38 autosomes. Observation on subsequent prophase stages showed that this large chromocenter represents the two positively heteropycnotic X-chromosomes in close association (Fig. 1). The close association of the two X-chromosomes in a positively heteropycnotic state is thus clearly in evidence at early and late prophase (Figs. 1 and 2). They were always associated end-to-end during this stage of the division cycle.

It is of considerable interest to note that in the case of prochromosome nuclei of spermatogonial cells, the positively heteropycnotic X- and Y-chromosomes were found as separate entities [4]. Thus, in contrast to the female, a single large heterochromatic body is not visible in prophase of spermatogonial cells.

The association of the two X-chromosomes was broken at prometaphase, although the X-chromosomes still stand out by virtue of their positively heteropycnotic nature (Fig. 3). At metaphase, the X-chromosomes were no longer readily recognizable since the autosomes at this time were also condensed (Fig. 4).

The fact that the association was observed between the two X-chromosomes of the female cell and not between the X and the Y of the male cell, although both were positively heteropycnotic, appears to favor the concept that attraction between the homologues is the cause of the somatic association, rather than a non-specific attraction between the heterochromatin.

Our previous findings on *Rattus norvegicus*, however, revealed that in this species, the totally heterochromatic X exists in a negatively heteropycnotic state until the onset of the first meiosis [5]. Accordingly, observation of prophase figures of the follicular cells of female rats has shown that the two X-chromosomes in a negatively heteropycnotic state were not in association with each other.

Only when the two X-chromosomes are in a positively heteropycnotic state do they appear capable of somatic association.

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THE FINE STRUCTURE OF THE FLAGELLAR APPARATUS OF *CHLAMYDOMONAS MOEWUSII*

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An electron-microscope examination of the fine structure of the flagellar apparatus of *Chlamydomonas moewusii* was carried out to support studies on the genetic control of motility in this flagellate [2]. These observations of sectioned material extend previous studies with shadowed whole mounts [3].

Methods.—Cells grown in bacteria-free culture [2] were fixed for 30 minutes in 1 per cent osmium tetroxide buffered with acetate-veronal to pH 7.6, washed, dehydrated quickly in an ethanol series, and imbedded in a mixture of 4 parts *n*-butyl and 1 part methyl methacrylate. Sections were cut on an experimental microtome [4] and observed with an RCA electron microscope, model EMU-2B.

Results.—Each flagellum consists of a cylinder of 9 double peripheral fibrils and a central pair, all imbedded in a structureless matrix and bounded by a membrane, about 100 Å thick, continuous with the plasma membrane of the cell (Fig. 1). Each individual fibril appears tubular and is 100–150 Å in diameter. The 2 central fibrils project distally about 200 mμ beyond the ends of the peripheral fibrils to form a mucronate tip to the flagellum. Within the cell, the 9 double peripheral fibrils continue to form the basal body of the flagellum. As in most Protista, a cross section through the basal body appears as an electron-dense ring comprising 9 denser areas (Fig. 6). A median longitudinal section of the basal region of the flagellum reveals an H-shaped structure not previously reported in other flagella or cilia (Fig. 4). This consists of an electron-dense cylinder, about 70 mμ in diameter and 120 mμ long, lying within the peripheral fibrils and traversed slightly below its middle by a diaphragm which extends across the flagellum. Fig. 3 shows a cross section through this central cylinder, presumably cut above the transverse diaphragm since the peripheral fibrils are still fairly distinct. The central fibrils appear to end unattached just above the distal end of the cylinder and do not continue into its lumen.

The paired flagella arise from opposite sides of an anterior papilla and pass to the anterior through a tunnel in the thickened cell wall. Along the median axis the two blepharoplasts join to share a common lumen (Fig. 5). Extending anteriorly into the papilla from the fused basal bodies is a thick-walled, bluntly-conical projection, whose lumen is at least partly separated from that of the blepharoplasts by one or two transverse partitions (Fig. 5). In some median sections a pair of double membranes, possibly indicating tubules or cisternae, are seen to arise together at the midpoint of the fused blepharoplasts and extend backward, one on each side of the contractile vacuole. No connection with the nucleus could be established.

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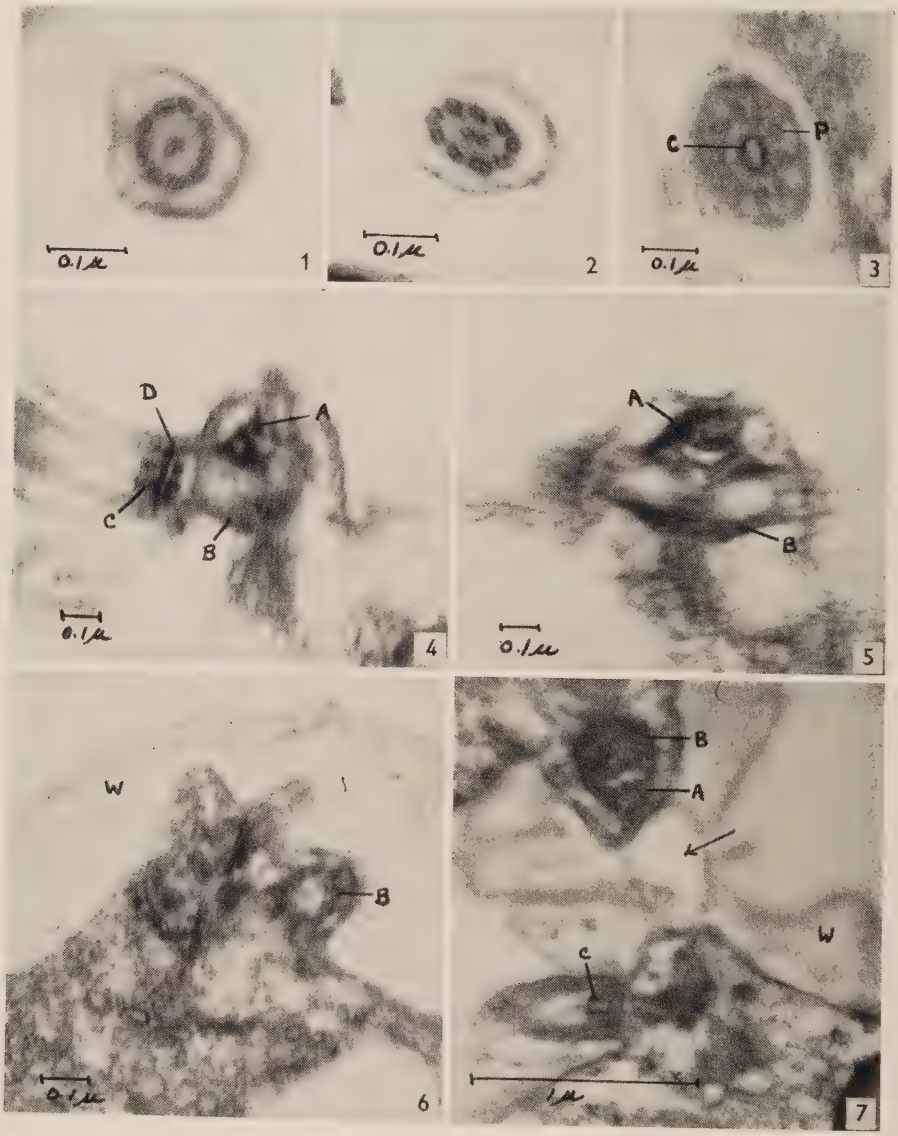

Fig. 1.—Cross section through a flagellum of a normal cell. × 106,000.

Fig. 2.—Cross section through a flagellum of paralysed mutant #1043. × 97,000.

Fig. 3.—Cross section through the central cylinder. × 72,000.

Fig. 4.—Median section through the central cylinder. × 51,000.

Fig. 5.—Median section through the fused basal bodies. × 51,000.

Fig. 6.—Oblique section across a basal body. × 63,000.

Fig. 7.—Median section through anterior regions of paired cells, 10 minutes after mating. Arrow indicates a bubble in the cell walls. × 30,000.

A, anterior projection of the fused blepharoplasts; B, basal body; C, central cylinder; D, transverse diaphragm; P, peripheral fibril; W, cell wall.

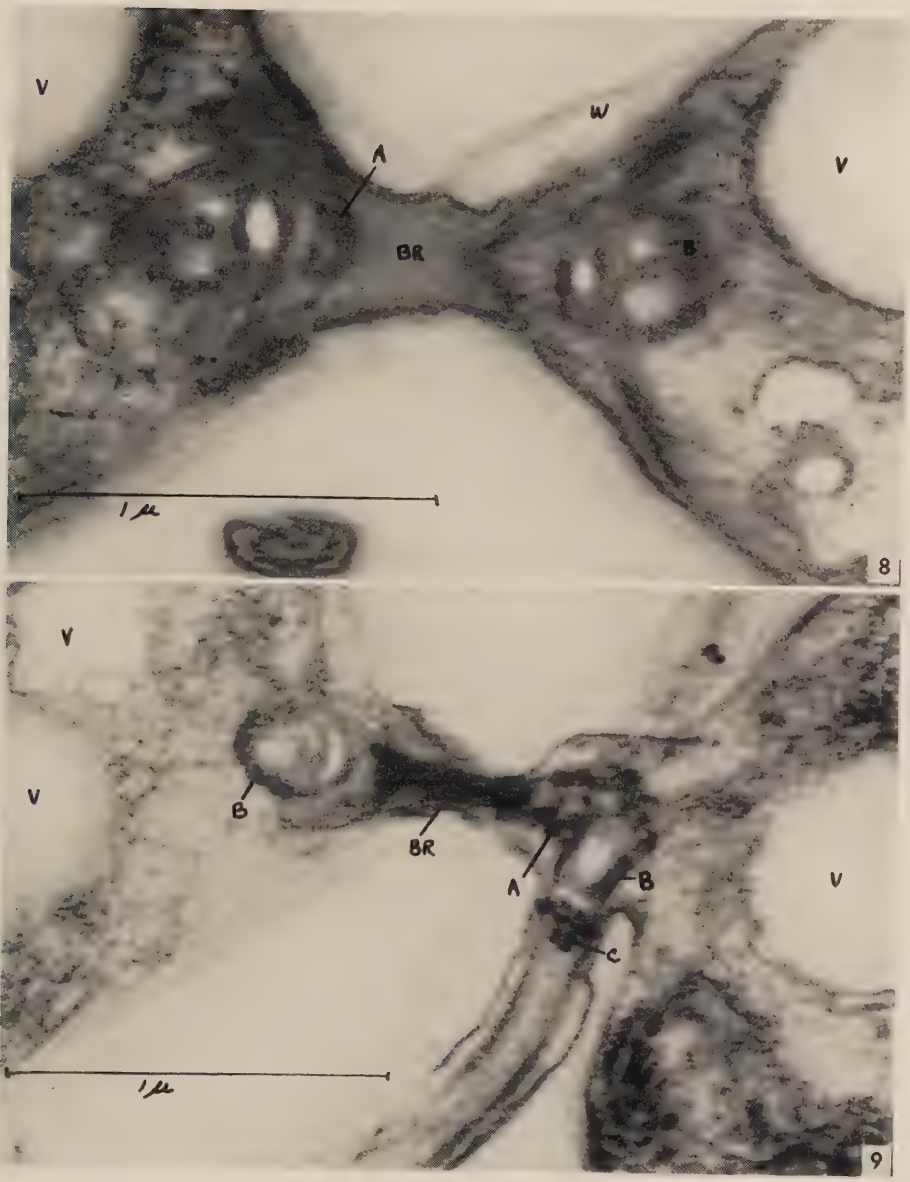

Fig. 8.—Median section through anterior regions of paired cells, 10 minutes after mating, showing protoplasmic bridge. × 55,000.

Fig. 9.—Median section through anterior regions of paired cells, 2 hours after mating. Note that flagellar planes are perpendicular. × 50,000.

A, anterior projection of the fused blepharoplasts; B, basal body; BR, protoplasmic bridge; C, central cylinder; V, contractile vacuole; W, cell wall.

The flagella of a series of mutants with impaired motility [2] were also examined (see Table I). No abnormal features other than that of length were observed with the electron microscope.

TABLE I. *Observations on the flagella of mutants of* Chlamydomonas moewusii.

Mutant number	Light-microscope observations	E-M observations
1002	Paralysed, recoverable in dikaryons (2)	Normal
1034, 1038, 1043	Paralysed, non-recoverable	Normal (Fig. 2)
1060	Paralysed, sterile	Normal
1085, 1087	Paralysed, flagella 2–4 μ long	Otherwise normal
224, 1083, 1084, 1086	"Flagellaless"	Flagella 0.6–1.0 μ long, otherwise normal

When cells of *plus* and *minus* mating types of *Chlamydomonas moewusii* are mixed under suitable conditions, the cells clump and ultimately pair. At first the cells are held together only by adhesion of their flagella, but within a few minutes a protoplasmic bridge is formed between the anterior papillae of the two gametes [1]. Since there is evidence that factors involved in flagellar motility are transferred from one cell to another through the bridge [2], cell pairs were fixed at various intervals from 5 minutes to 4 hours after mating and examined in thin sections. In pairs fixed 5 minutes after mating, no complete bridges were found, but tenuous extensions were occasionally observed projecting from the papillae of the cells. In one case the projections were seen to have met, but had not fused. Cell pairs fixed 10 minutes after mating were seen to be held together by a bridge of homogeneous protoplasm delimited by a well-defined membrane (Fig. 8). In cells mated for 1 to 4 hours, the anterior projections of the blepharoplasts appeared to have grown across the bridge and joined to form an electron-dense core to the bridge (Fig. 9). In the early stages of mating, bubbles were often found in the anterior region of the cell walls, which apparently break down by vacuolization to permit the formation of the protoplasmic bridge.

Summary. –The flagella of *Chlamydomonas moewusii* are similar to those of other Protista except for a small cylinder which lies within the peripheral fibrils in the basal region of the flagellum and is traversed by a diaphragm. The flagella of the 11 non-motile mutants examined display no abnormality in internal structure, though in some strains the flagella are only 0.6-1.0 μ in length. The protoplasmic bridge joining mated cells has an electron-dense core formed by extensions from the anterior projections of the blepharoplasts.

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RELATION OF THE RESPIRATORY COMPETENCE IN YEAST TO COLOR DEVELOPMENT AND RESISTANCE TO COPPER

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In the genetical studies of copper resistance in yeast, it is noticed that the resistant strains often develop brown color when grown in the presence of copper, while the sensitive strains in inhibited growth remain white [1, 2]. It was found, on the other hand, that copper induced respiration deficiency when normal (respiration-sufficient) strains were grown in sublethal concentrations of copper [6].

Five strains derived from a diploid baker's yeast (Fleischman) were used for comparative investigations of the color development on the nutrient media containing various concentrations of copper sulfate. The color development on tellurite and selenite media was also tested. These strains were rather strong in their inherent resistance to copper, as compared with wine yeast, tested before [1, 6]. They contained less than 1 per cent respiration-deficient cells in cultures grown under normal conditions.

These cultures developed a brown color when streaked or spread on an ordinary glucose-peptone nutrient agar containing 5×10^{-4}, 7×10^{-4} and 1×10^{-3} *M* copper sulfate, respectively. They showed intermediate color (various shades of tan) at 1.4×10^{-3} *M*. At 1.7×10^{-3} and 2×10^{-3} *M*, they appeared strongly inhibited and remained white, but showed survival above 50 per cent when plated quantitatively. On the other hand, previously isolated respiration-deficient subcultures from the above strains remained white on copper media, regardless of the copper concentration, although the degree of growth inhibition and survival was about the same as in the normal strains. It is apparent, therefore, that the brown color is developed only by the normal cultures growing in a certain range of copper concentration far below the lethal level. Even the normal cultures fail to develop color when the growth is strongly inhibited by the increased concentration of copper.

With respect to the reduction of triphenyltetrazolium chloride, which was found effective for the diagnosis of respiration deficiency [5, 6], normal cultures remaining white on a high concentration of copper also remained white when overlaid with tetrazolium agar (1 mg per ml of tetrazolium in molten 1.5 per cent agar, cooled to 50°C) as well as the respiration-deficient subcultures. Under such conditions, the tetrazolium overlay technique could not be used for the diagnosis of respiration deficiency. However, rather little increase in the frequency of respiration-deficient cells was found when these originally normal cultures remaining white on 1.7×10^{-3} *M* copper medium were suspended in water, plated on copper-free nutrient agar and scored by the tetrazolium overlay method. Even in the severely inhibited cultures on 2×10^{-3} *M* (below 0.5 mm in colony diameter as compared with 3 mm on copper-free medium), the frequency remained around 60 per cent. The rest of the cells were still respiratory competent after being transferred to copper-free medium. Table I shows a typical case of such a relation in the diploid baker's yeast. Parallel plating on 7×10^{-4} *M* copper medium showed that the frequency of colonies which failed to develop

brown color in spite of uninhibited growth was about the same as that of respiration-deficient colonies scored on the copper-free medium.

Summarizing the above observations, it is inferred that the ability to develop brown color on the copper medium depends upon the respiratory competence while the inherent degree of copper resistance is the same, irrespective of the respiratory competence and incompetence. The inability of copper-sensitive cultures to develop color on the copper medium is the result of impairment which was observed in the above-mentioned experiments at $1.7–2 \times 10^{-3}$ *M* copper. A recent investigation by Naiki [3] suggests that the brown pigmentation is due to the formation of copper sulfide in the cell. It may indicate a certain relation of the respiratory function to the reduction of sulfate to sulfide.

TABLE I. *Growth, color and frequency of respiration-deficient cells in a diploid culture as affected by the concentration of copper.*

Concentration (*M*) ...	7×10^{-4}	1×10^{-3}	1.4×10^{-3}	1.7×10^{-3}	2×10^{-3}	2.2×10^{-3}
Growth	*Confluent*		Increasingly inhibited		Severely inhibited	No growth
Color	*Brown*		*Tan to White*		*White*	—
Reduction of tetrazolium	*Strong*		*Weak*	*None*	*None*	—
Frequency of resp. def. cells (per cent)	<1	2	4	20	65	—

Development of black color by yeast cultures on the nutrient agar containing potassium tellurite (100 p.p.m.), and of reddish orange color on sodium acid selenite (200 p.p.m.) also showed a close correlation to respiratory competence. The normal cultures developed color in 24 hours of growth, while the respiration-deficient subcultures remained white. However, the contrast was weakened after 3 days as the latter become colored. Observations by Nickerson [4] on *Candida albicans* suggest a certain common relation in the reduction of tellurite, selenite and tetrazolium salt.

In several other cultures which produced respiration-deficient cells at very high spontaneous frequencies, color development on copper, tellurite and selenite appeared in various intermediate shades. But the color development seemed quite independent of the inherent degree of copper resistance and sensitivity.

It may be generally concluded that the color development on copper, tellurite, selenite and tetrazolium salt depends upon the respiratory competence where normal cells are allowed to undergo uninhibited growth. Such a condition in the case of copper is provided by the inherent resistance.

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LYSIS OF *BACILLUS SUBTILIS* BY AMINES, ACRIDINES, AND PHENOTHIAZINES[1, 2, 3]

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The ability of cysteamine and certain closely related derivatives to induce lysis of post-logarithmic phase cells of *Bacillus subtilis* has been reported [6]. Forty-three additional sulfur and/or nitrogen containing compounds consisting of selected amines, amino alcohols, amino acids, thio ethers, vitamins, pyrollidines, thiazoles, thiazolines, and thiazolidines were unable to induce lysis [6]. In subsequent unpublished work it has been found that in concentrations at which the cysteamine compounds are active, none of the common amino acids, water-soluble vitamins, purines, or pyrimidines possess lytic ability.

It had previously been observed that of nine clinically useful antimicrobial drugs tested in sub-bacteriostatic concentrations, only quinacrine could induce lysis of *B. subtilis* [5]. Inasmuch as amines such as cysteamine and acridines such as quinacrine are not ordinarily considered as bacteriolytic agents, it was believed to be of interest to test additional amines and acridines, as well as certain phenothiazines for possible lytic activity. The results are described in this communication.

The cultures used in this study consisted of a strain of *B. subtilis* that is lysed by cysteamine and by quinacrine, and a strain each of *B. macerans* and *Escherichia coli* whose cells are resistant to lysis by these compounds. The optimum conditions for the demonstration of lytic activity (exposure of metabolizing post-logarithmic phase cells contained in shallow shaken layers of nutrient broth to the test compounds for 24 hours [6] were employed in the present study).

The results of the tests for lytic activity with *B. subtilis* are presented in Table I. It may be noted that except for the two parent compounds, each of the acridines and phenothiazines were strongly active; and that also slightly more than half of the amines induced lysis. Of the active amines, however, only the dithiocarbamate and cysteamine were as efficient as the acridines and phenothiazines. None of the test compounds could induce lysis of *B. macerans* and only acriflavine (at concentrations above 3×10^{-3} *M*) could lyse *E. coli*. Inasmuch as organisms resistant to lysis by the active amines are also resistant to lysis by the acridines and phenothiazines, it is likely that the mechanisms of action of the three groups of lytic compounds are similar if not identical.

Of interest is the fact that several of the lytic compounds, including cysteamine, dithiocarbamate, tryptamine, tyramine, quinacrine, and chlorpromazine provide some degree of protection to living cells that are subsequently exposed to ionizing radiation [2, 3]. On the other hand, such non-lytic substances as histamine are, under some

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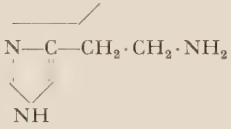
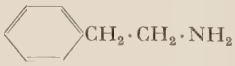
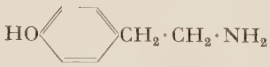
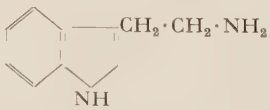
[3] Contribution nr 832 from the Department of Chemistry.

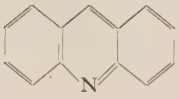
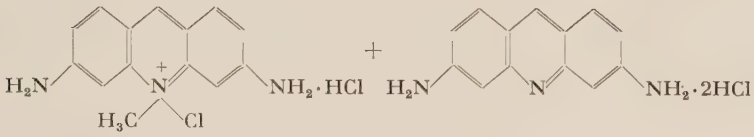
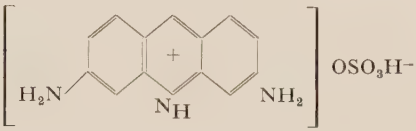
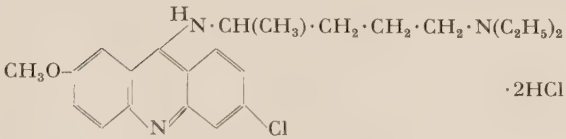
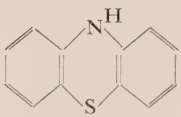
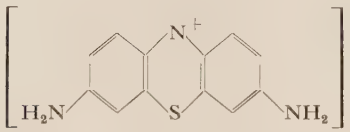
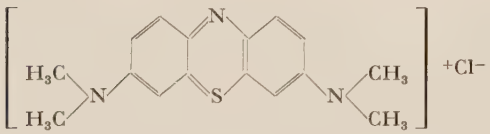
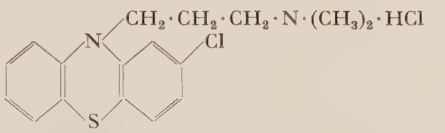
TABLE I. *Ability of the test compounds to induce lysis of post-logarithmic phase cultures.*[a]

Test compounds		Lowest active molar concentration[b]
AMINES		
Dithiooxamide	$H_2N \cdot C(S) \cdot C(S) \cdot NH_2$	1×10^{-2}
Sodium dimethyldithiocarbamate	$NaS \cdot C(S) \cdot N(CH_3)_2$	1×10^{-4}
Cysteamine	$HS \cdot CH_2 \cdot CH_2 \cdot NH_2$	5×10^{-4}
Isopropanolamine	$CH_3 \cdot CH(OH) \cdot CH_2 \cdot NH_2$	NON-LYTI
n-Butylamine	$CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2$	7×10^{-3}
sec-Butylamine	$CH_3 \cdot CH_2 \cdot CH(CH_3) \cdot NH_2$	1×10^{-2}
ter-Butylamine	$CH_3 \cdot C(CH_3)_2 \cdot NH_2$	1×10^{-2}
Putrescine	$H_2N \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2$	NON-LYTI
n-Amylamine	$CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2$	4×10^{-3}
Isoamylamine	$CH_3 \cdot CH(CH_3) \cdot CH_2 \cdot CH_2 \cdot NH_2$	4×10^{-3}
Cadaverine	$H_2N \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2$	1×10^{-2}
Glucosamine	$CH_2(OH) \cdot CH(OH) \cdot CH(OH) \cdot CH(OH) \cdot CH(CHO) \cdot NH_2$	NON-LYTI
Spermine	$H_2N \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2$	NON-LYTI
Spermidine	$H_2N \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2$	NON-LYTI
Prolamine		NON-LYTI
Histamine		NON-LYTI
n-Phenylethylamine		NON-LYTI
β-Phenylethylamine		3×10^{-3}
Tyramine		1×10^{-2}
Tryptamine		3×10^{-3}

[a] The compounds were added to fully-grown 16 hour cultures; the extent of lysis was measured 24 hours later in the Junior Coleman spectrophotometer model 6 (at 660 mμ).

[b] The minimum concentration that induced complete lysis within 24 hours' exposure of the cells to the compound. The highest concentration tested was 1×10^{-2} *M*.

TABLE I (cont.)

rotamine		NON-LYTIC
ACRIDINES		
cridine		NON-LYTIC
criflavine		3×10^{-4}
roflavine sulfate		1×10^{-4}
uinacrine		1×10^{-4}
PHENOTHIAZINES		
henothiazine		NON-LYTIC
hionin		1×10^{-4}
ethylene blue		1×10^{-4}
lorpromazine		4×10^{-4}

conditions, active as radiation protectors [2]. Obviously much additional investigation is needed to establish the relationship, if any, between the biochemical mechanisms of (*a*) radiation protection and (*b*) lysis of post-logarithmic cells of sensitive strains of *Bacillus*.

Several hypotheses concerning the mechanism of lytic action of the cysteamine compounds have been suggested [6]. It is possible, for example, that amines might be incorporated into enzymes responsible for cell wall synthesis and that such enzymes could no longer function properly. Amines inactive with *B. subtilis* might have a different spectrum of lytic activity. Thus although spermine and spermidine cannot induce lysis of *B. subtilis*, it is conceivable that the function of these compounds, present in large amounts in human semen [4] and in certain bacterial viruses [1], is that of contributing to the dissolution of the wall of the human ovum and of the host bacterium, respectively.

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POLOPLOIDY AND THE EFFECT OF ANAEROBIOSIS ON THE PRODUCTION OF RESPIRATORY DEFFICIENT YEAST[1]

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Subcultures from a large yeast colony produce large colonies of respiratory sufficient (AER) cells always accompanied by some small colonies of respiratory deficient (aer) cells [1, 4]. With few exceptions, small colonies produce only small colonies when plated out, indicating that the loss of respiratory activity in yeast cells is essentially irreversible. Evidence has been presented to show that this loss is generally caused by the irreversible damage of cytoplasmic granules [1].

In view of Warburg's hypothesis [6] that anaerobiosis may lead to the irreversible loss of respiratory activity by cytoplasmic granules and thus be a factor in the origin

[1] This work has been supported by a grant from the American Cancer Society.
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of cancer, Lindegren and Hino [3] made a study to determine if exposure of AER yeast cells to anaerobic condition could bring about loss of oxidative ability. Under carefully controlled conditions, they showed that the occurrence of respiratory deficiency was markedly accelerated under anaerobiosis as compared with aerobiosis. Harris [2] made a similar study using *Saccharomyces cerevisiae*, strain "Yeast Foam", and could not find significant change of the proportion of aer cells after incubation under aerobic and anaerobic conditions. He suggested that the process of respiratory loss may be independent of the presence of oxygen. The apparent contradiction between these two investigations is explained by the results of the following experiment.

TABLE I. *Counts of respiratory deficient (aer) colonies of various yeast strains after growth in nutrient broth under aerobic and anaerobic condition.*

Strain	Time in hours	Aerobic condition			Anaerobic condition		
		Total colonies	aer colonies	% aer	Total colonies	aer colonies	% aer
Haploid	0	2356	130	5.5			
15177	12	1225	45	3.7	1716	122	7.1
	24	706	24	3.4	1014	98	9.7
	36	348	4	1.2	458	48	10.5
	60	416	7	1.7	409	61	14.9
Haploid	0	2463	117	4.7			
13894	12	1018	57	5.6	1266	93	7.3
	60	467	17	3.6	668	56	8.4
	84	582	16	2.8	743	64	8.6
Haploid	0	885	516	58.3			
8256	12	364	71	20.9	587	88	15.0
	24	290	13	4.5	1215	86	7.1
	48	1184	38	3.2	2701	217	8.0
Diploid	0	897	32	3.6			
11296	12	173	2	1.2	875	11	1.3
	36	766	1	0.1	1137	11	1.0
	60	122	1	0.8	367	7	1.9
Diploid	0	925	3	0.3			
Baker's	14	1162	2	0.2	2727	7	0.3
F	24	1334	3	0.2	2094	11	0.5
	36	1607	8	0.5	1369	4	0.3
	60	1058	0	0.0	448	1	0.2
Tetraploid	0	1011	10	1.0			
	24	251	0	0.0	1244	1	0.1
	72	303	0	0.0	397	1	0.3

Cultures from the Carbondale breeding stock were grown on a glucose nutrient agar for 24 hours, the cells suspended in 10 ml of sterilized water to produce a slight turbidity and 1 ml of the suspension added to 10 ml of water for use as an inoculum. One ml of inoculum was added to cotton plugged test tubes (18 × 150 mm) and 50 ml Erlenmeyer flasks, both containing 15 ml of nutrient broth (glucose 20 g, peptone 3.5 g, KH_2PO_4 2 g, $MgSO_4$ 1 g, $(NH_4)_2SO_4$ 1 g, liquid yeast extract 3 ml, B-vitamin mixture 1 ml, water 1000 ml). The test tubes were placed in a one l anaerobic jar, nitrogen was passed through the jar for 30 minutes to remove air and 10 per cent pyrogallol was added to a small beaker of 50 per cent KOH solution (previously placed in the jar) to remove the remaining oxygen. During the anaerobic cultivation at 30°C, test tubes were taken out at intervals and after shaking, one ml of the culture solution was serially diluted with 6 ml or 10 ml water blanks and 0.1 or 0.05 ml of the dilution plated on the glucose nutrient agar. The tubes were replaced in the jar and anaerobic conditions were reestablished through the same procedures as before. Aerobic cultures were attained by placing the flasks on a horizontal rotary shaker (75 rpm). Temperature of cultivation and plating procedures were the same as with the anaerobic cultures. Plates were incubated at 30°C for 5 days and the number of aer small colonies and AER large colonies were counted on the plates containing less than 250 colonies. Since colony size alone is sometimes not a sufficient criterion for the differentiation between AER and aer cells, additional characteristics such as color of colonies [3] and ability (AER) or inability (aer) of growth on lactate agar [5] were used for differentiation when they were needed.

As is evident from Table I, the proportion of aer cells in total population is much higher in haploid cultures than in diploid cultures either under aerobiosis or anaerobiosis; in tetraploid, the proportion is still lower. It is also evident from the table that in haploid cultures there is a marked difference in the frequencies of aer cells after growth under aerobic and anaerobic conditions. Since the frequencies of aer cells are influenced both by relative growth rates of aer and AER cells and by the rate of the production of aer from AER, Lindegren and Hino [3] used a nutrient broth without carbohydrate, in which no multiplication of aer cells could take place. Using a haploid culture 8256, they showed that the rate of production of respiratory deficient cells from normal respiratory sufficient cells was actually increased under anaerobic condition.

With diploid cultures it is rather hard to draw a definite conclusion concerning the effect of anaerobiosis on the proportion of respiratory deficient cells, since the absolute frequencies of aer cells are so low. In general, diploid cultures produce about one per cent aer cells and hence they are not adequate materials for the experiments of this type. It is thus not surprising that Harris [2] could not detect the effect of oxygen on the occurrence of respiratory deficiency since he used a diploid culture.

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A SUBSTANCE FOR AQUEOUS DENSITY GRADIENTS

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In the determination of the specific gravity of cells and cell fragments, and also in the separation of similar objects by means of gradient centrifugation, it has always been something of a problem to find substances suitable for the building up of a satisfactory density gradient. During several years of work involving such problems, we have had the occasion to try a number of gradient substances, such as gum arabic, starch, proteins, polyvinyl-pyrrolidone, and thorotrast. They all turned out to be of limited usefulness, but none of them was really satisfactory over a broad range of applications.

Some years ago, therefore, we approached Dr. B. Ingelman of the firm "Pharmacia" and asked him whether he thought it possible to manufacture a substance specifically suited for gradient work. As desiderata, the following properties were specified: sufficiently high molecular weight to insure low osmotic activity even in highly concentrated solutions, sphaerical molecules to insure low viscosity, high solubility in water and weak salt solution, chemical inertness and stability to autoclaving, lack of toxicity, absence of nitrogen (so as not to prevent Kjeldahl analysis) — and finally, as high specific gravity as possible.

The substance prepared by "Pharmacia" meets most of these specifications and has in the last two years been tested in the Carlsberg Laboratory. The results of these tests have been so satisfactory that we wish to bring the product to the attention of other cell biologists.

The substance has been named "Ficoll" by its synthesizers, Dr. B. Ingelman and Dr. P. Flodin, who characterize it in the following terms: "Ficoll is a highly water-soluble, polymolecular, neutral colloid with properties similar to a polysaccharide. Its molecules are sphaerical. The colloid is stable in neutral and alkaline, non-oxidative solutions. Its aqueous solutions have a relatively low viscosity. The average molecular weight of the unfractionated substance is about 50,000. The commercial unfractionated product is not quite free of salt and should therefore be dialyzed or precipitated from aqueous solution by alcohol. Ficoll can be obtained from "Pharmacia Ltd., Copenhagen, Vanløse, Denmark".

In our laboratory we have used the product so described, after dialysis against distilled water, and therefore probably with a slightly higher average molecular size. 50 g Ficoll in 450 ml water were dialyzed 18 hours and the product dried, best by lyophilization. It is recommended not to concentrate the dialysate before lyophilization, as this increases the danger of breaking the flask, due to adhesion of the drying substance to the wall. Yield about 33 g. The densities obtained with the dialyzed product are about as follows:

g Ficoll dissolved in 2.00 g H_2O	$d\ ^{20°}_{4°}$
1.245	1.14
1.59	1.17
2.19	1.21

Even the most concentrated solution is not too viscous for pipetting.

In our laboratory Ficoll has so far been used for the determination of the specific gravity of amoebae and amoeba fragments, and in the centrifugation of amoebae and of yeast populations. The cells remain viable and undisturbed even after prolonged immersion. The details of these investigations will be published elsewhere. We have been informed that in other laboratories Ficoll has been used successfully on mammalian cells.

SYNTHÈSE D'ACIDE DÉSOXYRIBONUCLÉIQUE DANS LE CORPS PARABASAL DE *TRYPANOSOMA MEGA*[1]

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Reçu le 15 octobre, 1958

Les flagellates de l'ordre des trypanosomides possèdent, près de l'origine de leur flagelle, un corpuscule (appelé corps ou appareil parabasal par Wenyon [17] et par Grassé [7] et blépharoplaste par Doflein [5]), qui a déjà fait l'objet de nombreuses controverses. Les auteurs qui étudièrent les hémoflagellates au début du siècle furent frappés par son affinité pour les colorants nucléaires et par sa division caractéristique qui précède de peu la caryocinèse. Schaudinn [14] assimila cette structure à un second noyau. Adoptant le même point de vue, Hartmann [8] créa pour des protozoaires assez divers pourvu d'un tel "kinetonucleus" le groupe des binucléates. Cette théorie fut adoptée par Laveran et Mesnil [11] mais ne fut pas universellement admise et ses outrances la firent totalement rejeter [16, 6]. Vers 1925–30, l'application de la réaction de Feulgen, positive au niveau du parabasal, renouvela temporairement l'intérêt pour cette théorie des deux noyaux [3, 4, 10, 12, 13]. Cependant, cette notion semble être retombée dans l'oubli et les traités modernes ne la considèrent plus.

Nous avons repris le problème dans les perspectives actuelles en nous assurant d'abord de la présence ou de l'absence d'acide désoxyribonucléique dans le corps

[1] Alors que la présente note se trouvait chez l'éditeur, nous avons pris connaissance du travail de R. W. Horne et B. A. Newton sur *Strigomonas oncopelti*, publié dans *Exptl. Cell Research* **15**, 103 (1958). Nous apportons ici des arguments supplémentaires en faveur de certaines de leurs conclusions.

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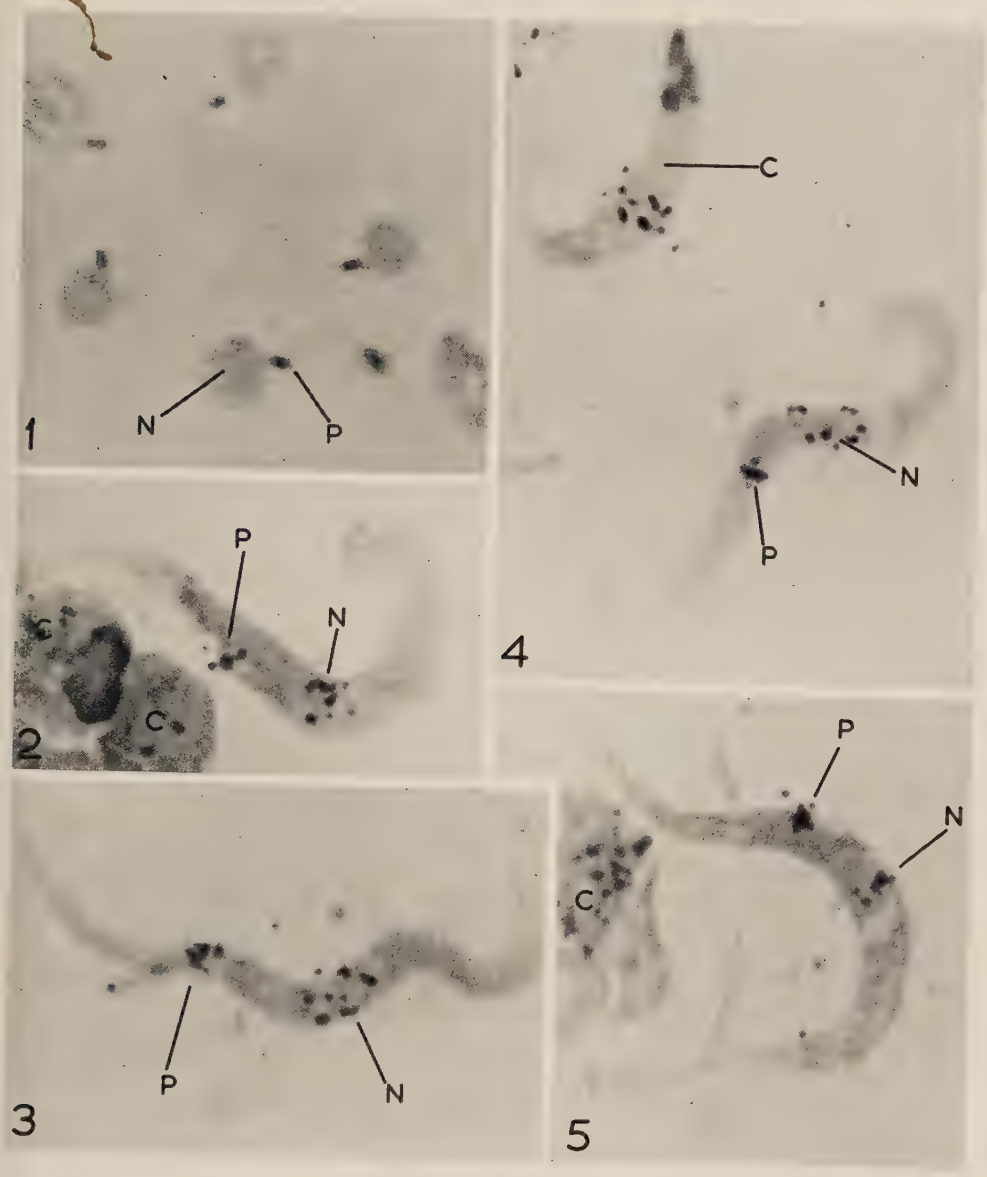

Fig. 1.—Crithidias de *T. mega* colorées au Feulgen. Seuls les noyaux (*N*) et les corps parabasaux (*P*) sont positifs.

Figs. 2 et 5.—Autoradiogrammes de trypanosomes marqués par la thymidine tritiée. Les noyaux (*N*) et les corps parabasaux (*P*) sont très distinctement marqués dans les formes trypanosomes. — Chez les crithidias (*C*), le corps parabasal est souvent trop rapproché du noyau pour permettre la définition de ces deux structures.

parabasal. Deux techniques différentes ont été utilisées chez *Trypanosoma mega* cultivé *in vitro* [2]: le contrôle à la désoxyribonucléase sur réaction de Feulgen et l'incorporation spécifique de thymidine tritiée mise en évidence par autoradiographie. Les résultats sont les mêmes sur la forme crithidia et la forme trypanosome. La localisation de la thymidine tritiée s'analyse plus clairement dans cette dernière forme, obtenue par addition d'urée au milieu [15]; en effet, la cellule est alors plus grande et son parabasal plus éloigné du noyau.

Réaction nucléale de Feulgen.—Comme on l'avait déjà vu, elle est plus intense dans le corps parabasal que dans le noyau (Fig. 1). Ni le noyau ni le parabasal ne sont colorés si l'hydrolyse acide est omise. De plus, nos observations montrent que les frottis incubés pendant 90 minutes à 37° avec de la désoxyribonucléase cristallisée perdent totalement la colorabilité au Feulgen tandis que dans des frottis témoins, incubés de façon semblable mais en l'absence d'enzyme, le noyau et le parabasal restent positifs.

Localisation de la thymidine tritiée par autoradiographie.—Après des incubations variables (de 3 heures à plusieurs jours) en présence de thymidine tritiée (15 μ c/ml), les trypanosomes furent étalés en frottis, fixés à l'alcool méthylique absolu et recouverts de l'émulsion Kodak A.R. 10. On constate sur les autoradiogrammes des trypanosomes incubés depuis plusieurs générations que le corps parabasal, comme le noyau d'ailleurs, est toujours fortement marqué par l'isotope radioactif (Fig. 2 à 5). Après des incubations moins longues, une partie seulement des individus ont incorporé de la thymidine. Après trois heures d'incubation, environ 25 % des noyaux et des parabasaux sont marqués. Toute radioactivité disparaît au niveau de ces deux structures lorsqu'on traite les frottis à la désoxyribonucléase.

Il n'est donc pas douteux que le corps parabasal contient et synthétise de l'acide désoxyribonucléique tout comme le noyau. De plus, on sait que cet organite est doué de continuité génétique. En effet, dans certaines espèces de trypanosomes, des souches aparabasales apparaissent spontanément ou sous l'action de l'acriflavine et le corps parabasal ne réapparait jamais dans ces souches [9] qui font ainsi penser aux souches achloroplastiques de certains phytoflagellates. Rappelons à ce sujet le rapprochement fait en 1917 par Alexeieff [1] et repris par Hoare [9] entre parabasal et "plastides" des phytoflagellates. Peut-être, en renouvelant ce parallèle, arriverait-on à une meilleure compréhension de ces particules douées de continuité génétique. Dans le cas du corps parabasal, à cette propriété s'ajoute celle de contenir et de synthétiser de l'acide désoxyribonucléique; la tentation de l'assimiler à un second noyau est donc très grande.

La présence éventuelle, dans d'autres espèces, d'acide désoxyribonucléique au niveau de structures extranucléaires capables d'autoduplication et sa signification sont à l'étude.

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LOCALIZATION OF NUCLEUS-SPECIFIC PROTEIN AS SHOWN BY TRANSPLANTATION EXPERIMENTS IN *AMOEBA PROTEUS*[1]

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In 1955, Goldstein and Plaut [1] presented evidence to show that, in *Amoeba proteus*, ribonucleic acid (RNA) was transferred from nucleus to cytoplasm—and in that direction only. That some degree of specificity was imposed on this transferred RNA was evident from the fact that the RNA was not transferred from one nucleus to another in a binucleate cell. Since it is probable that the RNA exists as a ribonucleoprotein in the cell, it appeared worthwhile to study the movement and localization of nuclear protein by the methods employed earlier. For example, one might find that the specificity imposed on the RNA was imparted by the protein moiety of the ribonucleoprotein.

The methods of nuclear transplantation, fixation, autoradiography, etc. are essentially similar to the methods described in detail in the earlier paper [1]. Briefly, the experimental procedure employed here was as follows: *Tetrahymena pyriformis* cultures were grown in a proteose-peptone solution with added ^{35}S-L-methionine; these labelled *Tetrahymena* were then fed to *A. proteus* for one day; the amoebae were then removed to a non-nutrient medium containing ^{32}S-L-methionine for 20 hours; subsequent autoradiography showed that these amoebae were fairly heavily labelled more or less uniformly throughout the cell.

Eight successful nuclear grafting operations were performed with these labelled amoebae. The nucleus from a labelled cell was grafted into an unlabelled cell that had *not* been enucleated. At various intervals between 4 and 70 hours following the operations, the amoebae were fixed on slides and exposed to autoradiographic strip-

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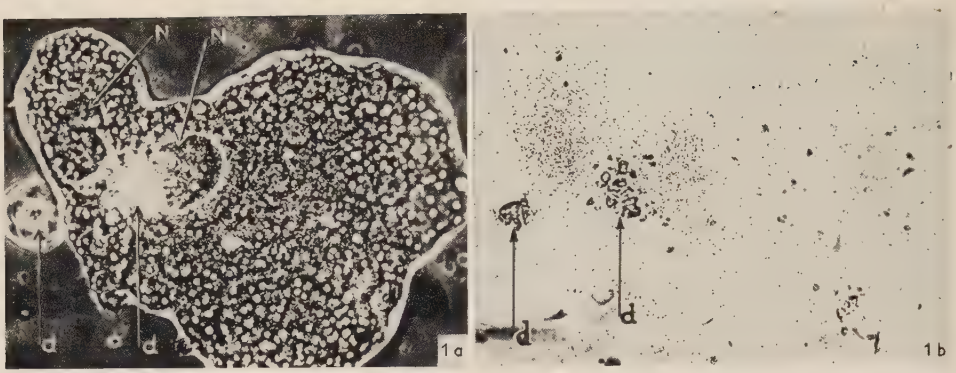

Fig. 1.—Fig. 1*a* shows phase contrast picture of fixed amoeba. Fig. 1*b* is bright-field picture showing autoradiograph. (*N*) indicates nucleus; (*d*) indicates debris (for purposes of orientation). This amoeba was fixed 70 hours following transplantation of ^{35}S-labelled nucleus. Both nuclei are well labelled. Cytoplasm is more heavily labelled than other amoebae in this series.

ping film. After 15 days exposure, the autoradiographs were developed and the specimens were examined by phase-contrast and bright-field microscopy.

The surprising observation, evident already at 4 hours after the operation, was that the label left the grafted nucleus to an appreciable extent and appeared mainly in the originally unlabelled nucleus (Fig. 1). Little labelling was evident in the cytoplasm. This observation was made in 8 out of 8 cases. With time, the originally unlabelled nucleus became somewhat more heavily labelled. These results are in sharp contrast to the observation on labelled nuclear RNA, in which case the label that leaves the nucleus appears only in the cytoplasm and not in the "host" nucleus.

The evidence clearly suggests that there is a nuclear protein(s) which migrates from nucleus to cytoplasm, remains there briefly, and shortly returns to the nucleus. That the label is in protein is seen from the fact that if it were leaving the nucleus as an amino acid, it would be incorporated into cytoplasmic protein to approximately the same extent as in the nucleus. This does not occur. Further evidence for this view comes from observations on nuclei grafted from amoebae that were exposed directly to ^{35}S-L-methionine and not subsequently exposed to non-radioactive methionine. Such nuclei apparently contain substantial amounts of unincorporated ^{35}S-L-methionine and when grafted to unlabelled cells the label diffuses into the cytoplasm. In such cases the label appears in significant amounts in cytoplasmic protein and especially in the food vacuoles where captured food organisms survive—and metabolize—for a time before being digested.

The surprising discovery of this rapidly migrating nuclear protein leads to considerable conjecture as to its role in the metabolism of the cell. One appealing hypothesis is that this protein is the carrier of nuclear RNA. The RNA synthesized in the nucleus may be conjugated with this protein for purposes of transport; the nucleoprotein would then move to the cytoplasm where the RNA would be deposited perhaps on the endoplasmic reticulum; then the protein would return to the nucleus for reutilization.

These observations provide support for two other viewpoints. First, they lend strong support to the contention that the nuclear membrane is freely permeable to molecules at least as large as proteins. Secondly, it seems highly unlikely that the nucleus can be a major (quantitatively) site of protein synthesis for the cell. However, this particular nuclear protein may account for the observations of Mazia and Prescott [2], made on *starved* amoebae, that the nucleus does make a significant direct contribution to cell protein synthesis as shown by comparison of ^{35}S-methionine uptake in enucleate and nucleate cells.

I am grateful to Doctor Daniel Mazia for fruitful discussions on this problem.

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PURINE STIMULATION OF WALKER CARCINOSARCOMA 256 IN CELL CULTURE[1]

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The effects of purines and pyrimidines upon tissue cultures were described as equivocal in a recent review [6]. Purine and pyrimidine bases apparently prolonged the survival time of chick embryo heart fibroblasts [7] but inhibited the growth of strain "L" mouse fibroblasts [2]. Hopkins and Simon-Reuss observed increased mitotic indices following treatment of chick fibroblasts with hypoxanthine but not with adenine [3]. Ehrensvärd *et al.* reported that hypoxanthine increased mitotic indices and the size of chick heart explants although adenine and guanine failed to do so [1]. The present communication describes a marked enhancement of growth of isolated Walker carcinosarcoma 256 cells by hypoxanthine, adenine and several derivatives of these purines.

The cells were grown in 60 × 15 mm Petri dishes according to the procedures of Puck [4, 9]. Cell suspensions for the inoculum were prepared from cultures of the tumor cells by treatment with 0.05 per cent trypsin in Earle's balanced salt solution for 3–5 minutes at room temperature. They were immediately diluted with whole basal medium. An initial number of 100–200 cells in 5.0 ml media were grown in

[1] This work was completed under Contract No. SA-43-ph-1886 with the Cancer Chemotherapy National Service Center, N.C.I., N.I.H., P.H.S.

duplicate Petri dishes containing 0–10 μg/ml of the test compounds. After 5 to 6 days at 37°C, the dishes were removed and the colonies were fixed with Bouin's fixative and stained with Giemsa stain. The colonies were counted, as were the cells in 2 colonies representative of 70 per cent of the colonies in each plate. The medium used has been described elsewhere [8]. It contained 14 essential amino acids and amino acid amides as well as glycine and serine, which are known to be stimulatory [5]. Also incorporated in the medium were a full complement of vitamins including 0.05 m*M* inositol, 0.2 m*M* each of pyruvate, oxalacetate, and α-ketoglutarate and 10 per cent dialyzed unheated bovine serum. The serum was dialyzed over a 24-hour period, with shaking at about 5°C against 2 changes of 100 volumes of modified Earle's balanced salt solution (containing 300 mg per cent glucose). The diluting salt solution for the medium was modified Earle's solution.

TABLE I. *Response of isolated Walker carcinosarcoma 256 cells to hypoxanthine, adenine and related compounds.*[a]

μg/ml ...	0	0.1	0.5	1.0	2.0	5.0	10.0
Compound				*Cells/Colony*[b]			
Hypoxanthine	153	102	76	148	538	393	670
Inosine	153	128	94	118	82	428	1134
Inosine-5′-monophosphate	153	142	200	228	210	482	1156
Adenine-sulfate	153	178	450	527	764	1348	1066
Adenosine	153	176	164	162	264	1390	1060
Adenosine-5′-monophosphate-dihydrate	153	128	168	266	240	792	828
Adenosine-5′-diphosphate-sodium salt	153	106	130	234	242	664	1244

[a] The basal medium is described in the text. Growth was for a period of 140 hours at 37°C.
[b] Average number of cells in 2 colonies representing the size range of 70 per cent of colonies. The cell numbers of the pairs usually agreed within 10 %.

Table I illustrates typical results obtained with hypoxanthine, inosine, inosine-5′-monophosphate, adenine, adenosine, adenosine-5′-monophosphate and adenosine-5′-diphosphate. An apparent plating efficiency of 80 per cent in the basal medium and as high as 100 per cent in the supplemented medium was obtained. The number of colonies counted was somewhat less at zero or low levels of purine additive than at higher levels possibly due in part to the small size of colonies which rendered counting difficult. The size of the colonies attained was much greater in media containing any one of the purine compounds at 5–10 μg/ml, although the cells grew at a moderate rate in the basal medium. The number of cells after 140 hours was as much as nine times that in the basal medium. Of the compounds tested, only adenine was appreciably active at less than 1 μg/ml. In addition, the adenine derivatives, adenosine

triphosphate, diphosphopyridine nucleotide and triphosphopyridine nucleotide exhibited stimulatory activity. Guanine, guanosine, deoxyguanosine, uracil, uridine triphosphate, cytosine, cytidine, cytidylic acid, thymine, thymidine, orotic acid, 5-amino-4-imidazolecarboxamide ribotide, xanthine, and uric acid in quantities up to 50 μg did not show growth-promoting effects. Combinations of the various active and inactive compounds did not reveal potentiation. Cells prepared directly from freshly excised tumor responded in a manner similar to subcultured cells.

Sato, Fisher, and Puck recently found that dialyzed serum which did not support growth of isolated HeLa cells recovered this capacity upon addition of cholesterol and coenzymes [10]. Similarly Neuman and McCoy found that addition of pyruvate, oxalacetate or α-ketoglutarate permitted rapid growth of isolated Walker carcinosarcoma 256 cells in media containing dialyzed serum [8]. The stimulation provided by the purine compounds is a further enhancement of growth since the purines did not replace the requirements of the isolated cells for the keto acids. In the initial studies of the growth-promoting properties of the keto acids, the isolated cells were grown for 7 days in a medium containing 15 per cent dialyzed serum. In the present studies, to demonstrate the further stimulatory effects of the purines, a medium containing 10 per cent dialyzed serum was utilized. The growth rates were sufficiently great for the production of large colonies in 5 to 6 days. The maximal average colony size obtained in the presence of purines (or derivatives) was 1390 cells, as compared to 153 cells in unsupplemented medium. The generation times calculated from these figures were 13.4 hours with purines and 19.3 hours in their absence.

In further studies, isolated cells grown in basal medium with and without added adenosine or inosine were incubated for different periods up to 140 hours. The number of cells in 20 randomly selected colonies in each petri dish was counted. The results revealed that the majority of colonies were derived from single cells, as indicated by the plating efficiency. These cells grew without an initial lag period regardless of supplementation. The growth rate was logarithmic and was the same for all plates for the first 48–72 hours. Thereafter, the growth rate of cells in the unsupplemented medium declined. However, the growth of cells in the medium containing adenosine or inosine continued logarithmically at an undiminished rate. Possibly limited quantities of essential substances are available from the serum. The effect of depletion of these substances can be relieved by the purine compounds. The tumor cells grew in a medium containing serum dialyzed for 2 weeks at 5°C against daily changes of 50 volumes of distilled water and were stimulated by adenosine. However, the growth rates were inferior to those attained with twice dialyzed serum.

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